Growth, development, and aging methods of the midget octopus, *Octopus huttoni*

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Abstract

This is the first study to be completed on the growth, developmental rates, and aging methods in the midget octopus, *Octopus huttoni.* Understanding life histories and population dynamics are important for fisheries management. Although *O. huttoni* may not be a commercial species, it is commonly caught as bycatch from oyster dredging suggesting that populations are heavily impacted. The overall aim of this study was to compare life history data from *O. huttoni* populations in Southern New Zealand. Morphometries and ages were compared between three populations with different habitats and substrate; Foveaux Strait (Bluff), Otago Harbour, and Offshore Otago. Morphometries were measured from 121 individuals, ages were estimated from 109 individuals using beak length, stylet weight, and beak and stylet increment analysis, and lipofuscin volume was calculated for 106 individuals. Paralarval young were also bred in captivity and reared at different temperatures to observe how growth changes with temperature. Aging methods were compared to determine the best method for wild-caught animals. Beaks provided the highest estimate, assuming that one increment is laid down daily, but are still suspected to be an underestimate because of feeding erosion. Stylets were validated to have daily growth rings but provided estimates that were much lower than beak estimates due to poor visualization of the nucleus and are therefore, not recommended as an accurate aging method. Lipofuscin accumulated slightly when compared to beak age, but the uneven distribution of granules may have resulted in increased variation as only 10 photos were taken per sample. Therefore, more photos (20-30) per sample and known-aged individuals should be used to establish a baseline trend. Individuals from the Foveaux Strait were found to be significantly smaller and younger than those found in the Otago Harbour. Those from Munida were smaller and younger than those from the Otago Harbour, but older than those from Bluff. These results support the ontogenetic shift hypothesis that paralarvae of merobethic species such as *O. huttoni* drift offshore during the planktonic stage, settle to the benthos, and then migrate inshore to spawn when mature. Since individuals from the Foveaux Strait were caught offshore, further studies should also collect octopus from inshore Bluff to compare and further test this hypothesis. The smallest

individual was estimated to be 28 days old, but *O. huttoni* are hypothesised to spend 40-70 days in the plankton before settlement. The lack of growth rings in laboratory reared paralarvae suggest that increments may start depositing only after settlement. Future studies should observe increments in known-aged, recently settled individuals and if this is true, that would indicate that beak estimates can only be representative of age post-settlement. Results from paralarval rearing confirmed that at lower temperatures, embryonic development is slower, and that mother size and age does not influence offspring size. Overall, this comprehensive study provides vital life history information for three different populations of *O. huttoni* in Southern New Zealand.

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3RL	third, right arm length
AL	arm length
BIA	beak increment analysis
CV	coefficient of variation
HL	head length
HW	head width
LWS	lateral wall surface
ML	mantle length
MW	mantle width
PAS	periodic acid-Schiff
PML	Portobello Marine Laboratory
PVC	Polyvinyl chloride
RSS	rostrum sagittal section
SIA	stylet increment analysis
TL	total length
WW	wet weight

Chapter 1: General introduction

1.1 Octopus in New Zealand

Octopus have a global distribution from the poles to the tropics, and from the shallows to the abyss (Wood & O'Dor, 2000). They are key predators of the benthos, but also prey for pelagic species making them an essential part of the oceanic food chain (Higgins *et al.*, 2013; Wood & O'Dor, 2000). In coastal Southern New Zealand, there are two common species of octopus; the common Māori octopus *Macroctopus maorum* Hutton, 1880 and the midget octopus *Octopus huttoni* Benham, 1943 (O'Shea, 1999; Stranks, 1996). As adults they are easily distinguishable as *M. maorum* is much larger (up to 248 mm in mantle length) than *O. huttoni* (up to 57 mm in mantle length) (Carrasco, 2014). In juveniles however, the anatomy is so similar, that there is often confusion between the two species (O'Shea, 1999). The easiest way to distinguish between the two species is by the presence of a large papilla on the tip of the mantle and two iridescent patches located preocular on the dorsal side of the mantle in *O. huttoni* individuals (O'Shea, 1999) (Fig. 1.1). Juvenile *O. huttoni* can be identified by the single row of black chromatophores down the arms in addition to the presence of the iridescent patches (O'Shea, 1999) (Fig. 1.2).

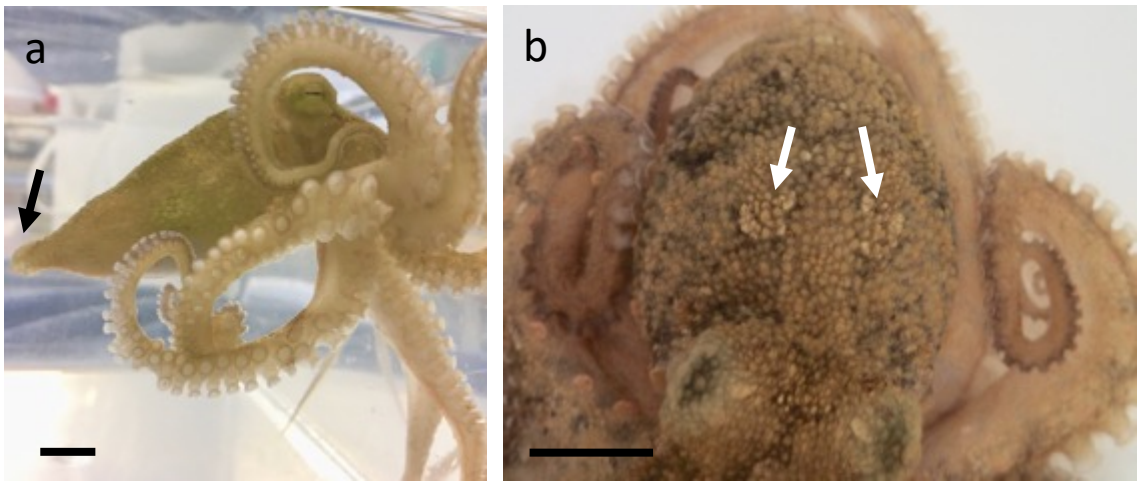

Figure 1.1. Example of (a) papilla on the tip of the mantle (black arrow) and (b) iridescent patches (white arrows) on the dorsal side of mantle of live *Octopus huttoni* male. Scale bars = 10 mm.

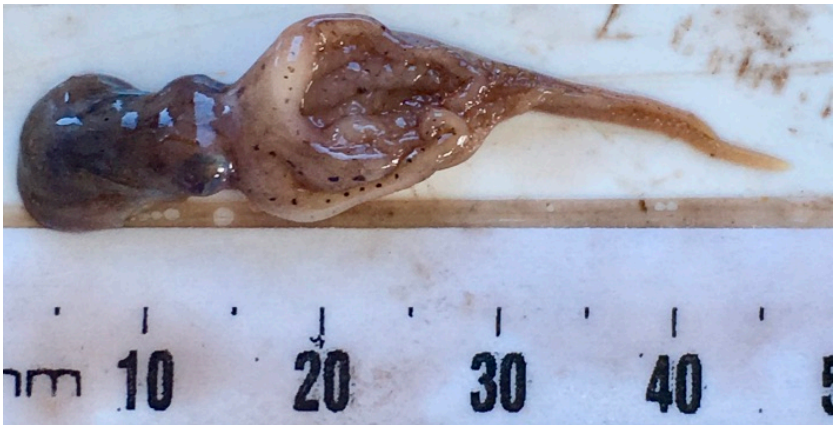

Figure 1.2. Example of the rows of black chromatophores which run down each of the arms in juvenile *Octopus huttoni*.

1.2 Ecology of the midget octopus

The midget octopus *O. huttoni,* also previously known as *Octopus warringa, Robsonella australis*, and *Robsonella huttoni* is a small bodied, long armed species of octopus with a mantle length of up to 57 mm and a total length of 236 mm (Carrasco, 2014; O'Shea, 1999; Dell, 1952). It is found along the continental shelf and upper slope from 0 to 386 m deep and is a merobenthic species, with a planktonic paralarval stage which is predicted to last about 40-70 days (Stranks, 1996; Carrasco, 2014). Due to its planktonic stage, *O. huttoni* has a relatively wide distribution from the Auckland Islands, to the tip of the North Island, to the south east of Australia (O'Shea, 1999; Stranks, 1996) (Fig. 1.3). This wide distribution proves that *O. huttoni,* as a species, can withstand a wide range of temperatures (Higgins *et al.*, 2011).

Midget octopus are often caught as dredge bycatch in oyster and scallop fisheries where the animals live amongst the oyster shells (Sam Heenan, Pers. observ.). As they are not a commercial species, these bycatch incidences are unreported, and no studies have assessed the effects that the dredging may have on the individuals and local population.

Figure 1.3. Distribution of *Octopus huttoni* Benham 1943 across Australia and New Zealand, represented as the black circles, the synonym *Octopus warringa* Stranks 1990 as the hollow circle, and *Octopus berrima* Stranks and Norman 1992 as the black triangles. *Octopus berrima* is a species of the same octopus type (Type 2). Each shape represents individual specimens collected from that point (Adapted from O'Shea, 1999).

1.3 Growth rates

Growth rates and size at maturity are crucial parameters in understanding the life history and population dynamics of any species (Bettencourt & Guerra, 2001; Lourenço *et al.*, 2015). These data are also important for managing a fishing stock. As the octopus fishery continues to increase in other parts of the world and the numbers of bycatch continue to go unreported, life history information is even more crucial (Higgins *et al.*, 2013; Lourenço *et al.*, 2015; Doubleday *et al.*, 2006; Barratt & Allcock, 2010). In comparison with other marine species, cephalopods have been largely understudied with respect to life history traits. Currently, published data regarding growth rates in octopus is mainly limited to *Octopus vulgaris* (Hernández-López *et al.*, 2001; Barratt & Allcock, 2010; Raya & Hernández-González, 1998; Perales-Raya *et al.*, 2014b, 2014a; Sanchez *et al.*, 1998; Perales-Raya *et al.*, 2010; Franco-Santos *et al.*, 2016; Hermosilla *et al.*, 2010;

Semmens *et al.*, 2004), *Octopus pallidus* (Doubleday & Semmens, 2011; André *et al.*, 2009; Leporati *et al.*, 2008b; Doubleday *et al.*, 2006), *Octopus maya* (Villegas-Bárcenas *et al.*, 2014), and *M. maorum* (Doubleday *et al.*, 2011).

Octopus are known for having indeterminate growth and terminal spawning. As a terminal spawner, individuals grow to maturity, reproduce and then die either after copulation (males) or after caring for their eggs (females) (Moltschaniwskyj & Carter, 2010). Indeterminate growth patterns are known for their exponential initial increase in body size, which switches to a logarithmic growth trend, and reaches an asymptote as the animal becomes an adult (von Bertalanffy, 1957; Moltschaniwskyj & Carter, 2010; Lourenço *et al.*, 2015). Although studies have shown indeterminate growth in other octopus species such as *Octopus cyanea* (Van Heukelem, 1973), *Octopus mimus* (Cortez *et al.*, 1999b) and *Robsonella fontaniana* (Uriarte *et al.*, 2010), none have examined growth in the midget octopus, *O. huttoni*. Size is typically correlated with age to get a size-age estimate of growth (André *et al.*, 2009; Forsythe & Hanlon, 1988), but indeterminate growth in octopus makes this difficult to standardize. Furthermore, growth rates may vary between populations due to different habitat structure and food availability, therefore affecting overall growth (Cortez *et al.*, 1999b; Uriarte *et al.*, 2012; Boletzky, 1994). Temperature/seasonality and food availability are the main factors affecting growth in cephalopods (Forsythe & Van Heukelem, 1987; Vidal *et al.*, 2002; Ramos *et al.*, 2014). This is especially true in the paralarval stage as they are more sensitive to their environment and require a lot of energy to support rapid growth (Semmens *et al.*, 2004).

1.4 Temperature and growth rates

Temperature is known to have an effect on the growth rates of a variety of marine organisms, and low temperatures are often associated with slower development and growth (Sanford *et al.*, 2006; Herwig *et al.*, 2012; Ramos *et al.*, 2014; Hatfield, 2000). Larvae in particular are extremely sensitive to temperature change (Higgins *et al.*, 2011). For example, Sanford *et al.* (2006) have shown that zoea larvae of Atlantic fiddler crab, *Uca pugnax,* from the colder part of their range grow quicker

than larvae from a warmer population when reared at the same temperature. This phenomenon is called counter gradient variation, where individuals from high-latitude populations grow faster than those from low-latitude populations when grown at the same temperature (Noyola *et al.*, 2013; Sanford *et al.*, 2006; Conover *et al.*, 1990).

Octopus are known for having rapid growth and short life spans for their body size, but they also have strong life-history plasticity which allows them to adapt quickly to their environment (Doubleday *et al.*, 2016). This may give them an advantage in our warming oceans because they could gradually adapt before the temperature increase becomes detrimental. Doubleday *et al.* (2016) has found that over the past 60 years, cephalopod abundance has generally increased. This leads researchers to believe that they will thrive within the near future with the increase in ocean temperature while many other species suffer.

Studies have observed how temperature affects the growth rates in cephalopods, specifically, *Octopus joubini, Octopus bimaculoides,* and *Loligo forbesi* (Forsythe & Hanlon, 1989; Forsythe, 1984; Forsythe & Hanlon, 1988; Wood & O'Dor, 2000). Forsythe and Hanlon (1988) found that when kept at 23 °C, the life span of *O. bimaculoides* was shortened by 20% and 5-month-old individuals were three times larger than individuals raised at 18 °C. During the time of the study (spring to early summer) temperatures can range from 14°C to 19°C (Forsythe & Hanlon, 1988). This indicates that their life span was shortened but they grew quicker than those reared in colder water. In the squid *L. forbesi*, a 1°C increase in temperature during the exponential growth phase caused individuals to be three times heavier at 90 days post-hatching (Forsythe & Hanlon, 1989). These studies provide evidence that temperature is an important factor for cephalopod growth.

1.5 Aging methods

Many cephalopod studies have shown that the statolith, a calcareous element enabling balance and orientation, can be used for aging due to daily growth increments (rings) (Liu *et al.*, 2015; Jackson, 1989; Villanueva, 2000). However, the

lines on statoliths in octopods are not as clearly visible as in squid species (Jackson, 1989; Doubleday *et al.*, 2006, 2011), therefore, a new method of aging octopus is needed in order to fully understand the life history of this group. Methods such as stylet (internal shell) increment analysis (Fig. 1.4) (Doubleday *et al.*, 2006, 2011; Leporati *et al.*, 2008b; Hermosilla *et al.*, 2010), beak (feeding apparatus) increment analysis (Fig. 1.5) (Perales-Raya *et al.*, 2010, 2014a) and age-pigment lipofuscin (Fig. 1.6) (Doubleday & Semmens, 2011) have been tested, but only on other species of octopods such as *O. pallidus* (Doubleday *et al.*, 2006; Leporati *et al.*, 2008b; Doubleday & Semmens, 2011), *O. vulgaris* (Hermosilla *et al.*, 2010; Perales-Raya *et al.*, 2010, 2014a), *Octopus tetricus* (Ramos *et al.*, 2014; Leporati & Hart, 2015), and *M. maorum* (Doubleday *et al.*, 2011).

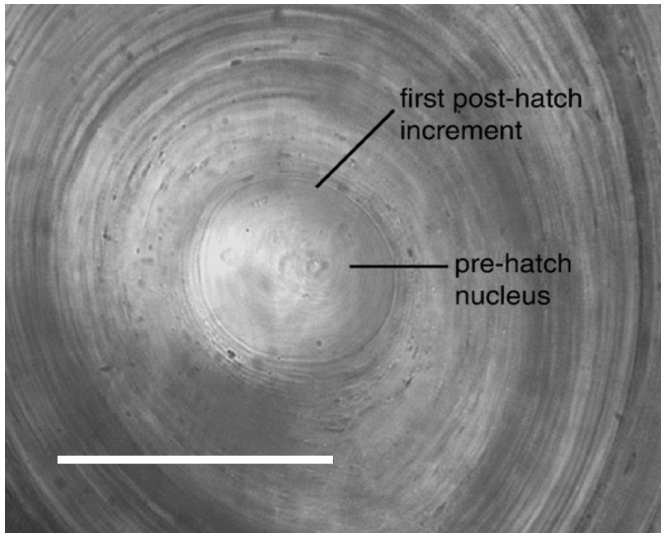

Figure 1.4. Typical microstructure of a transverse section of a laboratory-raised *Octopus pallidus* stylet and the location of the pre-hatch nucleus and the first post-hatch increments. Scale bar = 50 μm (Adapted from Doubleday *et al.,* 2006).

Figure 1.5. Increments on the posterior region in the beak of *Octopus vulgaris*. Scale bar = 200 μm (Adapted from Perales-Raya *et al.*, 2010).

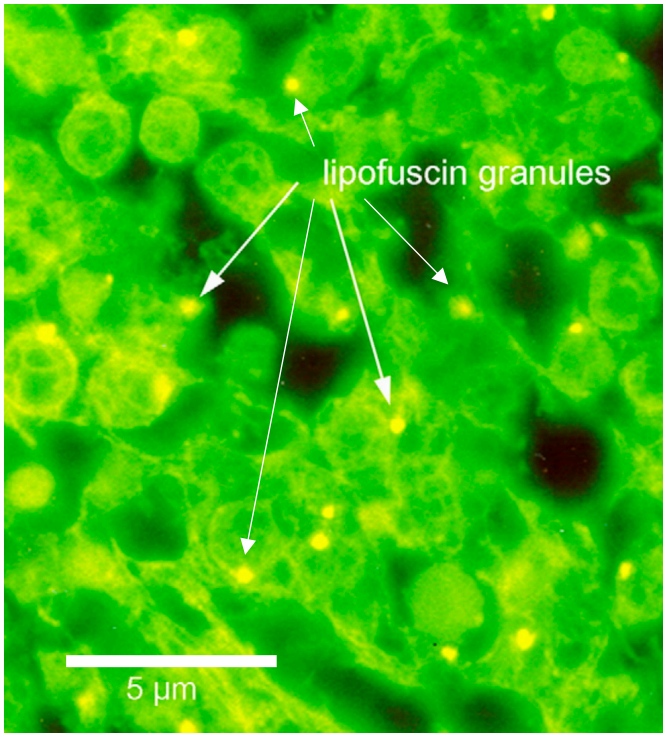

Figure 1.6. Lipofuscin granules in the optic lobe of *Macroctopus maorum* at 1250x magnification prepared by histology methods. Scale bar = 5 µm (Adapted from Doubleday & Semmens, 2011).

Stylet, paired hard parts found in some groups of octopus that is thought to be the remnant of an ancestral mollusc shell, and beak increment analysis involve counting the growth rings that are laid down in them. In some species of cephalopod such as the squid *Loligo vulgaris* (Villanueva, 2000), *Sepioteuthis lessoniana* (Jackson, 1990), and *Idiosepius pygmaeus* (Jackson, 1989), and the octopus *O. vulgaris* (Hermosilla *et al.*, 2010) the assumption of daily increments has been validated through periodic fluorescent staining of the statolith or stylet. This is done by either using calcein (a fluorescent stain) or tetracycline (an antibiotic), which leaves a visible reference in hard parts (Jackson, 1989; Villanueva, 2000; Jackson, 1990; Hermosilla *et al.*, 2010). It has been observed that the number of increments between each stain mark equals the number of days passed between staining (Jackson, 1989; Villanueva, 2000; Bettencourt and Guerra, 2001; Hermosilla *et al.*, 2010), validating the deposition of one increment per day in those species.

Many studies have also used increments on cephalopod beaks to estimate age (Clarke, 1965; Raya & Hernández-González, 1998; Perales-Raya *et al.*, 2014b,

2014a; Villegas-Bárcenas *et al.*, 2014; Hernández-López *et al.*, 2001; Liu *et al.*, 2015). This is similar to stylet increment analysis, but is hypothesised to be an underestimate as the increments are worn away from the friction caused by feeding (Perales-Raya *et al.*, 2010).

Another developing aging method for octopus involves lipofuscin. Lipofuscin is an age-pigment that accumulates over time in the lysosomes and has been used to age a variety of invertebrates including crustaceans (Maxwell *et al.*, 2007; Sheehy, 1992, 1990b, 1989, 1990a) and echinoderms (Du *et al.*, 2013) because of its non-degradable nature. It occurs in all metazoans from the oxidation of polyunsaturated fatty acids and therefore is correlated with metabolism (Doubleday & Semmens, 2011; Zielinski & Portner, 2000). Because lipofuscin is an end product of respiration, it is assumed that the relationship between the amount of lipofuscin and age would be linear if respiration rates are reasonably consistent. This has been seen in crustacean brains such as the American lobster, *Homarus americanus*, (Wahle *et al.*, 1996) and the crayfish *Cherax quadricarinatus* (Sheehy, 1990a), but in the one octopus species studied, *O. pallidus*, an exponential trend was observed (Doubleday & Semmens, 2011). This can be attributed to cephalopods having high levels of oxidative damage and low levels of enzymatic antioxidant defence over time (Zielinski & Portner, 2000; Doubleday & Semmens, 2011). Lipofuscin can be analysed by either counting pigment granules in stained histological sections or measuring fluorescent intensity of either histological sections under a fluorescence microscope or the dissolved age pigments with a spectrofluorometer (Hammer & Braum, 1988).

1.6 Aims

Presently there are no studies on growth or aging of the midget octopus *O. huttoni.* This thesis aimed to examine the trends in growth and development of this species from three different locations by estimating age and comparing morphometries. Locations included the Otago Harbour, Foveaux Strait, and Otago continental shelf hereby referred to as Otago, Bluff, and Munida respectively as the Offshore Otago site was on the research transect named the Munida transect. Otago Harbour

(Otago), Foveaux Strait (Bluff), and Offshore Otago (Munida) were chosen as locations because of their differences in substrate type/habitat and depth. Otago Harbour contains muddy/sandy substrate, Foveaux Strait is comprised of oyster beds, and Offshore Otago is comprised of bryozoan reefs. Otago and Bluff are both affected by anthropogenic activities as Otago is affected by dredging to widen the harbour channel and Bluff is affected by dredging from the oyster fishery. These different habitats could indicate that populations of *O. huttoni* living there are of different sizes and therefore grow at different rates. Three main different aging methods were compared to investigate the efficiency of each method; (1) stylet increment analysis, (2) beak increment analysis, (3) lipofuscin volume ratio. The disposition of daily increments in *O. huttoni* stylets were also validated in the laboratory by staining with tetracycline and counting increments from known-age paralarvae. Size measurements were then compared to age to investigate how size at age differs between locations. Finally, eggs were brooded and paralarvae were grown in captivity to study embryonic development and growth.

Marine animals from different populations have been known to have different growth rates, morphometries, etc therefore, these data were compared between populations from Otago, Bluff, and Munida (Sanford *et al.*, 2006; Hatfield, 2000; Ramos *et al.*, 2014; Herwig *et al.*, 2012).

This thesis aims to address the following:

- To investigate the differences in morphometries, age, and condition between *Octopus huttoni* from three locations in Southern New Zealand (Otago, Munida, and Bluff) (Chapter 2 and 3).
- To compare three different aging methods (1. Stylet increment analysis, 2. Beak increment analysis, and 3. Lipofuscin volume ratio) (Chapter 3).
- To use estimated age to model size at age growth (Chapter 3).
- To investigate the use lipofuscin as an aging method in *Octopus huttoni* (Chapter 4).
- To determine how temperature affects growth and survivability of *Octopus huttoni* paralarvae (Chapter 5).
- To document embryonic development from live *Octopus huttoni* eggs laid and brooded in captivity (Chapter 5).

Chapter 2: Morphology and growth of *Octopus huttoni*

2.1 Introduction

2.1.1 Growth in cephalopods

Needham (1964) defines growth as "an increase in size, whereas development is the sum of growth and differentiation". Information on growth and reproduction is crucial for understanding an organism's life history and for managing a fish stock (Doubleday & Semmens, 2011; Doubleday *et al.*, 2006, 2011). The next two chapters will be focusing on growth of the midget octopus, *Octopus huttoni*. Thus far, information on O. huttoni is limited to thermal tolerances and preferences (Higgins *et al.*, 2011) and the description of the eggs and paralarvae (Brough, 1965; Carrasco, 2014). There is a variety of biotic and abiotic factors that affect growth of an organism including food availability, competition, sex, size, age, temperature, light availability, salinity, and oxygen (Forsythe & Van Heukelem, 1987). Of these, the most important factors are size, temperature, and food availability (Forsythe & Van Heukelem, 1987; Vidal *et al.*, 2002; Ramos *et al.*, 2014) and as global climate change continues, it is critical to study how growth will be affected (Doubleday *et al.*, 2016).

Growth rates can provide information on individual condition, environment, population dynamics, and resource availability (Moltschaniwskyj, 2004). The rate of growth is measured as the change in biomass or length over time and when size-at-age data are available, we can begin to understand how this rate changes as a function of age, condition, and biomass (Moltschaniwskyj, 2004). While growth in other marine organisms such as fish has been studied in depth, studies on cephalopod growth are relatively new.

Typically, cephalopods have short life spans with rapid, non-asymptotic growth (Semmens *et al.*, 2011; Forsythe & Van Heukelem, 1987; Moltschaniwskyj, 2004; Leporati *et al.*, 2008b). Most cephalopods are semelparous, in which they grow to maturity, reproduce, and die (Pecl *et al.*, 2010; Semmens *et al.*, 2004; Moltschaniwskyj & Carter, 2010; Boyle & Boletzky, 1996; Franco-Santos *et al.*, 2014; Forsythe & Van Heukelem, 1987). Cephalopods are also generally,

indeterminant in growth meaning their high plasticity allows them to vary their growth trajectories depending on environmental conditions (Moltschaniwskyj & Carter, 2010, 2013; Pecl, 2004; Sebens, 1987).

Many studies have found some species of octopus to have two-phase growth (Leporati *et al.*, 2007; Cortez *et al.*, 1999b; Semmens *et al.*, 2011, 2004; Forsythe & Van Heukelem, 1987). The two-phase growth curve typically includes an early rapid exponential growth phase followed by a slower logarithmic (power) growth phase (Forsythe & Hanlon, 1988; Forsythe & Van Heukelem, 1987; Semmens *et al.*, 2011, 2004; Cortez *et al.*, 1999a; Uriarte *et al.*, 2009). One reason growth is exponential in the beginning of octopod life may be because the paralarvae go through a non-growth phase immediately after hatching before starting to grow. This was seen in *Octopus maya* as the hatchling's weight did not differ significantly in the first 10 days post hatching although they were feeding. After this non-growth period, weight increased the next 20 days (Moguel *et al.*, 2010). This was replicated with the same species, for which Rosas *et al*. (2014) found a 15-day post hatch non-growth phase. After 15 days, an exponential growth phase was discovered as in other studies (Rosas *et al.*, 2014). Uriarte *et al*. (2009) suggested that the exponential growth phase in *Robsonella fontaniana* starts during embryonic development, but more studies need to be done on other species to determine when growth begins.

2.1.2 Allometric growth

One way that growth can be visualised is by using allometry. Allometry is "the study of size and its consequences" (Gould, 1966). In biology, allometry is used to determine the differences in size proportions by observing changes in morphometries (Gould, 1966). There are two main types of allometric relationships; weight-length and length-length. Weight-total length relationships can be an indicator of condition in teleost fish, squid, and cuttlefish, but not octopods. Octopus lack skeletal hard parts, such as vertebra in fish and pens in squid, which prevents reliable total length measurements (Moltschaniwskyj, 2004). Mantle length-weight relationships can be used instead of total length as

the mantle retains its size and shape in contrast to the arms which can break, shrink, and stretch.

The power function $y=ax^b$ is known as the equation of simple allometry and is used to graph the relationship between length-length and weight-length (Gould, 1966; Derusha *et al.*, 1987; Uriarte *et al.*, 2009; Hernando *et al.*, 2009; Forsythe, 1984). In the case of a weight-length relationship, if *b* is greater than 3 (positive allometry), then the weight (y) is increasing faster than length (x) (Ricker, 1979). If *b* is less than 3 (negative allometry), then the weight is increasing slower than length. In the rare cases where $b=3$ (isometry), both variables are growing at the same rate (Gould, 1966; Ricker, 1979; Sangun *et al.*, 2007; Villanueva, 1995). Typically in weight-length comparisons, the exponent *b* lies between 2.5 and 4.0 (Forsythe & Van Heukelem, 1987). In the case of a length-length relationship, the same applies as in weight-length except instead of 3, 1 is used because 3-dimensions (weight) are no longer used. The trend is isometric when $b=1$, positively allometric when $b>1$, and negatively allometric when $b<1$ (Gould, 1966; Ricker, 1979; Villanueva, 1995).

In previous allometric studies on octopus, some have been found to have length-weight relationships that are isometric, and some allometric. In a few octopus species such as, *Octopus bimaculoides* (Forsythe & Hanlon, 1988) and *Octopus vulgaris* (Guerra, 1979), the exponents for length-weight relationships are all near 3 indicating isometric growth. However, *Octopus briareua*, *Octopus joubini*, and *Octopus digueti*, display allometric growth for all or parts of their life cycle (Forsythe & Hanlon, 1988; Forsythe & Van Heukelem, 1987). Allometry can tell us how the species grows over its lifetime and prompts us to start to think about why this is.

2.1.3 Aims

Understanding growth leads to understanding productivity, population dynamics, and how a species may respond to global climate change (Doubleday *et al.*, 2016). This chapter aimed to investigate size variation, condition, and growth of three

different populations of wild-caught *O. huttoni* in Southern New Zealand and compare current data to unpublished data from 2004-2006. Otago, Munida, and Bluff represent three different habitats with possibly different populations of *O. huttoni* of different sizes and therefore different growth rates. The objective was addressed by comparing morphometries and length-length and weight-length allometric relationships between locations. Data on temperature and feeding rates were also collected to understand how temperature affects feeding and therefore growth.

2.2 Methods

2.2.1 Animal collection

Approval from the University of Otago Animal Ethics Committee was obtained (AEC No. 59/17) in order to collect, keep, manipulate and euthanise *O. huttoni* for the duration of this project.

A total of 59 midget octopus were collected as dredge bycatch from oyster fishers and NIWA survey transects in the Foveaux Strait, Bluff from August 2017 to October 2018 (Table 2.1 & Fig. 2.1) and then transported to Portobello Marine Lab (PML). Of these 59, 32 were caught dead and stored frozen. The remaining 27 were collected live and transported to PML the same day to be settled in tanks (Table 2.1). Of these 27, 13 died in the tanks shortly after transport and the remaining 14 were kept for staining and breeding, which will be discussed in chapters 3 and 5 respectively.

A total of 61 live *O. huttoni* were captured in homemade tube traps and dredges from the Otago region from August 2017 to September 2018. This includes seven from a bryozoan reef offshore of Otago, three from the beach at PML, 49 from tube traps placed off of the PML wharf, and five from the Dunedin wharf (Table 2.1 & Fig. 2.1). The offshore Otago site is herein referred to as “Munida” as the site was located along a monthly offshore transect termed “the Munida transect”. Otago animals were caught using traps made with polyvinyl chloride (PVC) pipes,

weights, and rope tied from the PML wharf and Munida animals were caught by a dredge.

Table 2.1: Sites, dates, and depths where *Octopus huttoni* was collected.

Latitude	Longitude	Depth	Location	N (sample size)	Date sampled
-46.688056	168.158611	-33m	Foveaux Strait: Site 1	59	August and October 2017, January, March, and October 2018
-46.680983	167.981166	-40m	Foveaux Strait: Site 2		
-46.739283	168.261051	-31m	Foveaux Strait: Site 3		
-46.742350	168.408867	-24m	Foveaux Strait: Site 4		
-46.720017	168.474183	-18m	Foveaux Strait: Site 5		
-45.786183	170.923183	-90m	Munida	7	September and November 2017
-45.828114	170.640347	-3m	Portobello wharf	49	September 2017-September 2018
-45.880752	170.506226	Surface	Dunedin wharf	5	September 2017 and September 2018

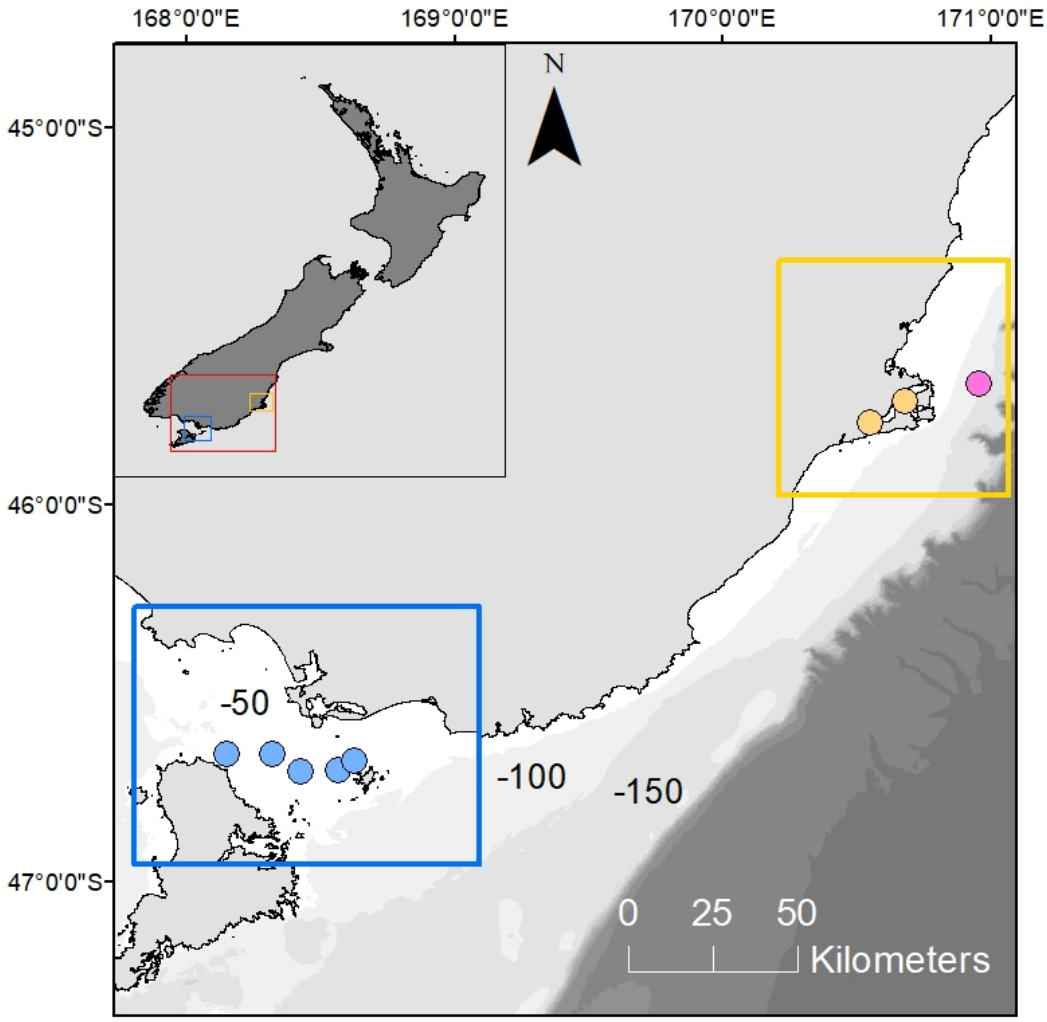

Figure 2.1. Map of the two Otago sites (orange dots), five Bluff sites (blue dots), and Munida site (pink dot) where *Octopus huttoni* were collected. The yellow square indicates the Otago region and the blue square indicates the Bluff region. The small numbers indicate maximum isobath depth for that shaded portion. Each shade increases 50 meters in depth.

2.2.2 Animal husbandry

Adults were housed in black, 55 L, fiberglass tanks in the tank room at PML with 350 μm mesh covering the inflow and outflow pipes and a weighted plexiglass lid with foam edges to prevent escapes. Flow rate was set to 2.5 L per minute with zero bubbles as excess aeration can cause death if bubbles are caught under the mantle. A variety of PVC tubes, rocks, shells, and jars were added to the tanks to give the animals a choice of hiding places and reduce stress. Checks were carried out three times a day (0900, 1700, and 2200 h) by PML staff and every other day by myself. Laboratory technicians checked for flow and deaths whereas I cleaned

crab carcasses, mesh filters, checked for individual preference in hiding location, and added food to ensure each octopus always had access to five crabs. Location of the octopus in the tank and how many crabs were eaten since the last check was recorded. No more than two octopus were housed in one tank with the exception of juvenile octopus received from Bluff where two tanks housed four each. More than two could be held in each tank because smaller juveniles that had recently settled did not need as much space as adults. Temperature loggers placed in the tank room at PML were taking recordings every 30 minutes to record the temperature of the water flowing into the tanks. Temperature was plotted against the average number of crabs eaten per day to determine how temperature affected feeding rates.

When an octopus was no longer needed alive, they were humanely euthanised using a lethal dose of Aqui-S®, in which clove oil is the primary component, and placed in a freezer for at least 24 h.

2.2.3 Measurements and data collection

All 121 frozen octopus were thawed in water prior to dissection. Morphological data collected before dissection included wet weight (WW) (g), total length (TL)(mm), mantle length (ML)(mm), mantle width (MW)(mm), head length (HL)(mm), head width (HW)(mm), arm length (AL)(mm), 3rd right arm length (3RL)(mm), sex, and number of arms intact as this can effect WW (Fig. 2.2). Lengths were measured with calipers and recorded to the nearest 0.5 mm and weight was recorded to the nearest 0.01 g. Males were identified by locating the hectocotylus, or penis arm, on the third, right arm (Fig. 2.3a) and if it looked like a normal tentacle with suckers down to the tip, then the sample was labelled as female or juvenile. Sex was confirmed by macroscopically observing the gonads inside of the mantle.

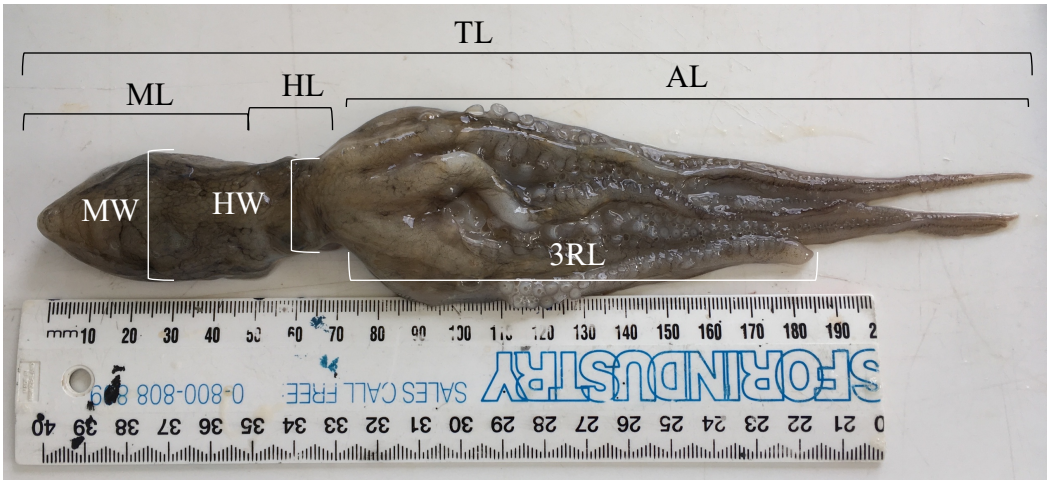

Figure 2.2. Locations of morphometric measurements; TL (total length), ML (mantle length), AL (arm length), HL (head length), MW (mantle width), HW (head width), and 3RL (third right arm length) demonstrated on a male, adult *Octopus huttoni*.

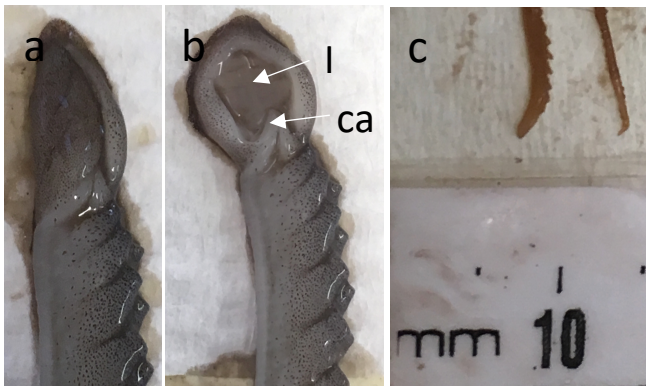

Figure 2.3. Photos of mature male's hectocotylus (penis arm) (a) closed and (b) open with white arrows indicating the lingua (l) and calamus (ca) and (c) pubescent male *Octopus huttoni* with a normal tentacle on the right for comparison.

Morphometries from the present study were compared to those collected from 2004-2006 and dissected in 2008 to 2010 (Fiona Higgins unpublished data). Only Bluff specimens were compared as all except two from the 2004-2006 dataset were from Bluff. Specimens from 2004-2006 were preserved in formalin whereas 2017-2018 were fresh frozen. This should not have affected the results because preservation does not cause morphological deformation of coastal octopus (Voight, 1991). The 2004-2006 samples were sedated with 7.5% magnesium chloride (MgCl) and then euthanised by freezing. They were then placed flat in formalin the day after euthanasia to allow the MgCl to keep the muscles relaxed and preserved until dissection date. Animals were preserved from 9 to 1427 days before measurement and dissection.

2.2.4 Analyses

Principal component analyses (PCA) were done using Primer 6 (version 6.1.13) to visualize distribution of data between locations, year-datasets, and sex. For each factor (location, year, and sex) two PCAs were run; one with all of the measured morphometries and one with calculated ratios (%ML/TL, weight/ML, MW/ML, and HW/HL).

Shapiro-Wilk goodness of fit normality tests were done in JMP 11.0.0 on all morphometries and ratios (WW, TL, ML, MW, HL, HW, %ML/TL, WW/ML, MW/ML, HW/HL) for Otago, Bluff, and Munida as well as for the 2004-2006 dataset and the 2017-2018 dataset. Whenever the data were found to be unevenly distributed, logarithmic transformations were done. If the transformations did not aid normal distribution, the non-transformed data were used instead and non-parametric Chi-squared or Kruskal-Wallis tests were run. If significant, Steel-Dwass post-hoc tests were done to further investigate the differences. These tests were done on 250 specimens from the 2004-2006 dataset (248 from Bluff and 2 from Otago) and 88 from the 2017-2018 dataset (41 from Bluff, 44 from Otago, and 3 from Munida). Samples which were too damaged (eg: missing arms) or had missing data were excluded from these analyses. This included eight specimens from 2004-2006 and 21 from 2017-2018.

To test for weight-length and length-length relationships between Otago and Bluff and between the 2004-2006 and 2017-2018 datasets, all data were log transformed and WW was plotted against ML and TL was plotted against ML. In the weight-length relationship, log (WW+1) and log (ML+1) was used as some log (WW) values were negative. Power curves where $y=ax^b$ were fit to each set of data. The exponent b was then used to determine if the data fit an allometric ($b \neq 1$ or 3) or isometric (b=1 or 3) relationship. The root mean squared error (S_b) was then calculated for each curve and a t-test was then done using the statistic $t_s=(b\text{-}(3 \text{ or } 1))/S_b$ to determine if b is significantly different from 1 (length-length) or 3 (weight-length) (Sangun *et al.*, 2007; Morey *et al.*, 2003). If b was equal to 1 (length-length) or 3 (weight-length), growth would be determined isometric and if not, growth was determined allometric.

2.3 Results

2.3.1 Morphometries between sites

In the PCA of all of the measured morphometries, total length was the main factor driving PC1 (0.637) and PC2 (0.486) and mantle length was the main factor driving PC3 (-0.728). In this PCA, PC1 explained 81.9% of the variation. In the PCA of just the Bluff measured morphometries, weight was the main factor driving PC1 (0.855) and PC2 (0.509) and head width was the main factor driving PC3 (0.647). In this PCA, PC1 explained 86.8% of the variation.

In the PCA of the morphometric ratios, the main driving factors were %ML/TL for PC1 (0.856), MW/ML for PC2 (0.846), and both MW/ML and HW/HL for PC3 (0.500). In this PCA, PC1 explained 66.7% of the variation and PC2 explained 30% of the variation. In the PCA with only the Bluff morphometric ratios, the main driving factors were weight/ML for PC1 (-0.997), HW/HL for PC2 (-0.693), and %ML/TL for PC3 (0.454). In this PCA, PC1 explained 73.2% of the variation and PC2 explained 13.6% of the variation.

There were visible differences in measured and ratio morphometrics between locations and datasets, but not sex (Fig. 2.4).

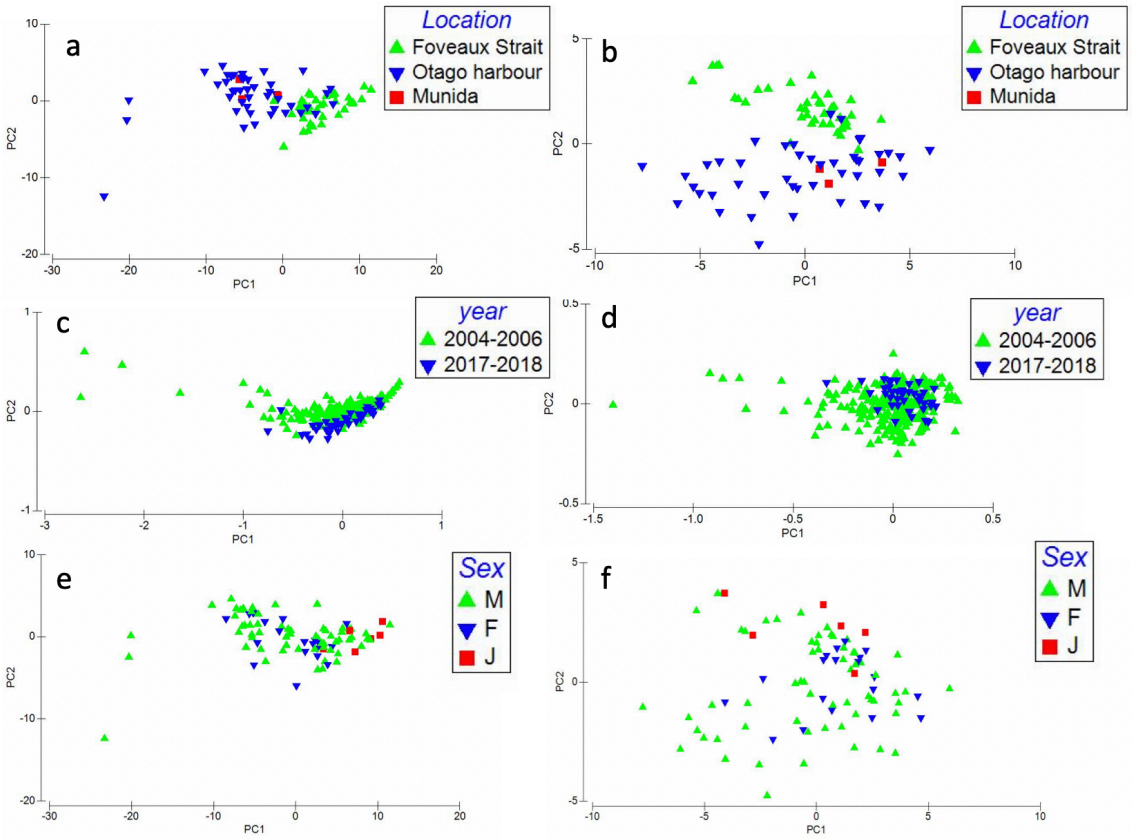

Figure 2.4. Principal component analysis by location (a and b) (Otago n=44, Munida n=3; Bluff n=32), year-dataset (c and d) (2004-2006 n=250; 2017-2018 n=32), and sex (e and f)(male (M) n=60, female (F) n=19, and juvenile (J) n=8) with (a, c, and e) morphometric measurements of *Octopus huttoni* (weight, total length, mantle length, mantle width, head width, and head length) as variables and (b, d, and f) calculated ratios (% mantle length to total length (%ML/TL), weight/mantle length (weight/ML), mantle width/ mantle length (MW/ML), and head width/ head length (HW/HL)) as variables.

Not all morphometries were normally distributed between sites, year-datasets, and sexes and log transforming the data did not aid normality. Therefore, non-transformed data were used to run non-parametric Wilcoxon, 1-way Chi-squared tests. All measurements between individuals from different sites (Bluff, Otago, and Munida) were significantly different (χ^2 (1, N=338), $p<0.0001$). Otago individuals were larger than those from Bluff and Munida individuals were either similar in size to Otago individuals or to both Otago and Bluff individuals (Table 2.2). All measurements and %ML/TL were significantly different between the year-datasets (χ^2 (1, N=291), $p<0.02$) except for WW (χ^2 (1, N = 291) = 0.006, p=0.939). Individuals caught in 2017-2018 were larger except in HW, and %ML/TL in which those caught in 2004-2006 were significantly larger. Males were significantly

larger than females in regard to all measured morphometrics (χ^2 (1, N=338), p<0.0041). Calculated ratios such as W/ML, MW/ML, and HW/HL were not significant between year, location, nor sex (p>0.0937).

Table 2.2. Steel-Dwass results for measured morphometries of *Octopus huttoni* from different locations (Otago, Munida, and Bluff). Different letters signify sites that are significantly different in that measurement.

Measurement	**Otago**	**Munida**	**Bluff**
Weight	A	AB	B
Total Length	A	AB	B
Mantle Length	A	AB	B
Mantle Width	A	AB	B
Head Length	A	A	B
Head Width	A	AB	B

2.3.2 Feeding rates with temperature

Feeding rates were highest in the beginning of December and February thru the beginning of April (Fig. 2.5). These high rates in feeding matched peaks in temperature that occurred during this time (Fig. 2.6). There was a dip in feeding rates during summer between January and February (Fig. 2.5).

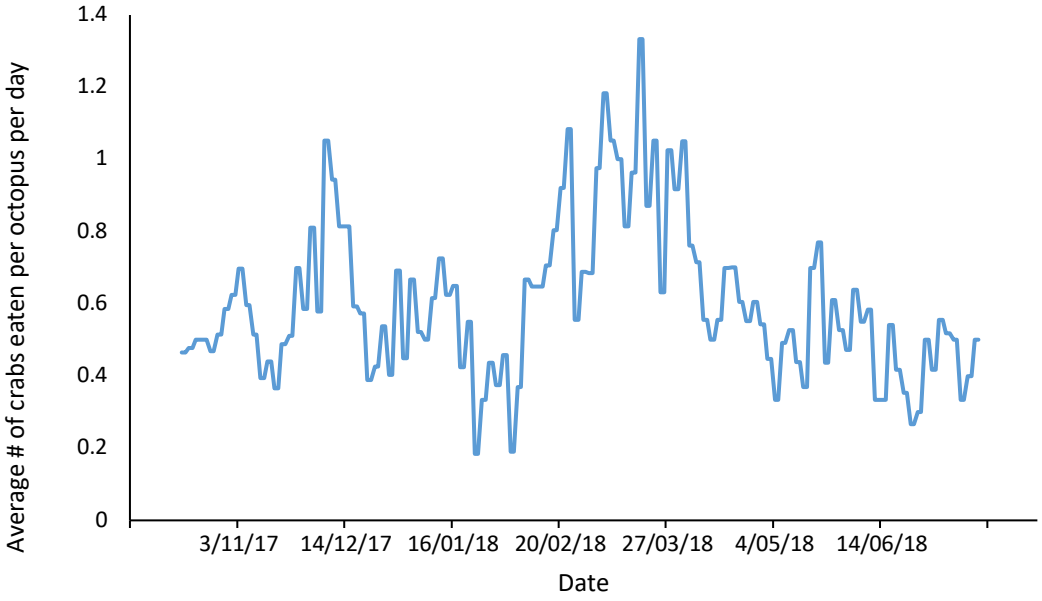

Figure 2.5. Average number of crabs eaten per *Octopus huttoni* per day over 10 months.

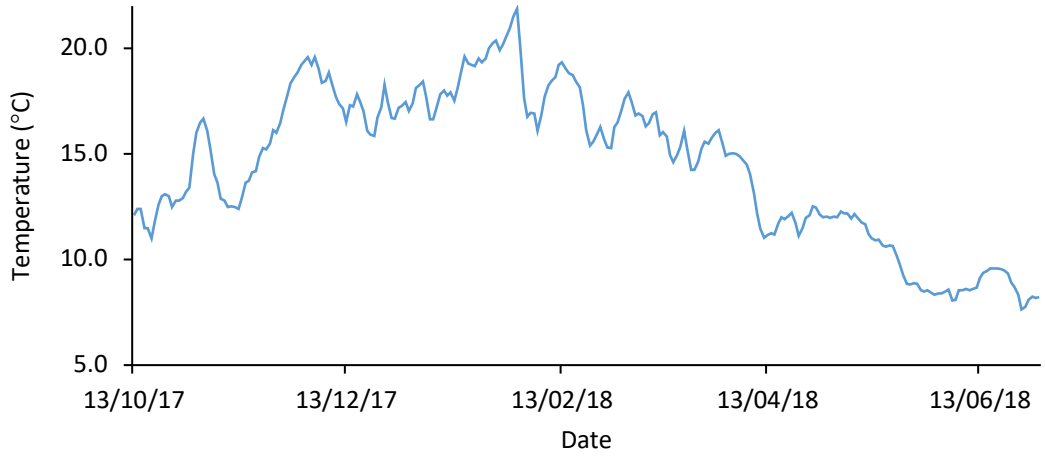

Figure 2.6. Tank water temperature (°C) at Portobello Marine Lab over 9 months.

2.3.3 Weight-length and length-length relationships

There was a negative allometric relationship between WW and ML of individuals from Otago (b=2.3335, t-test, p=1.268 x 10^{-5}, df=46) and individuals from Bluff (b=2.8112), but the t-test showed that b was not significantly different from 3 (Bluff t-test, p=0.526, df=40) (Fig. 2.7).

The relationship between WW and TL appeared to be positively allometric (b=3.2628), but the t-test showed that it was not significantly different from 3 (t-

test, p=0.094, df=46) (Fig. 2.7). There was also a positive allometric relationship between WW and TL of individuals from Bluff as the *b* exponent was significantly different from 3 (*b*=4.8679, t-test, p=1.957 x 10^{-21}, df=40).

Individuals from both year-datasets showed a positive allometric relationship between WW and ML but they were both not significantly different from 3 (2004-2006, *b*=3.2243, t-test, p=0.156, df=247) (2017-2018, *b*=3.0482, t-test, p=0.136, df=87) (Fig. 2.8).

There were positive allometric relationships between WW and TL for both year-datasets where *b* values were significantly higher than 3 (2004-2006, *b*=4.4509, t-test, p=1.204 x 10^{-21}, df=247) (2017-2018, *b*=4.7958, t-test, p=4.661 x 10^{-20}, df=87) (Fig. 2.8).

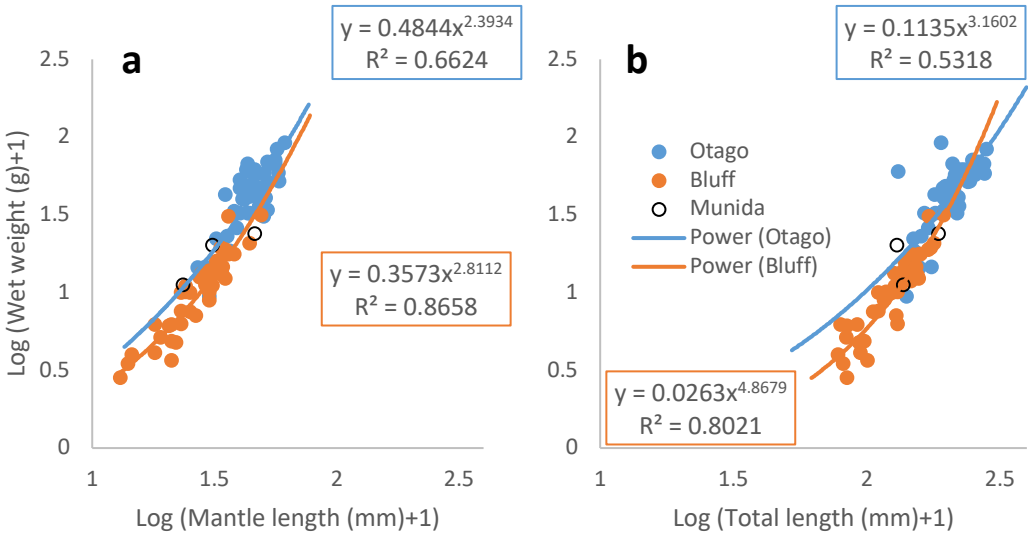

Figure 2.7. (a) Weight-mantle length and (b) weight-total length relationships from *Octopus huttoni* collected from Otago (n=44), Bluff (n=41), and Munida (n=3).

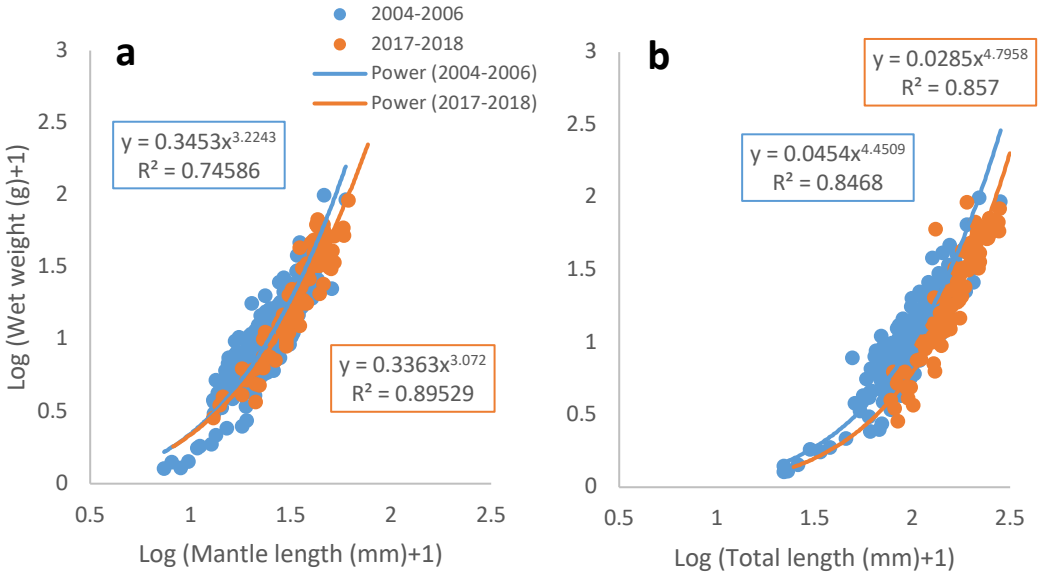

Figure 2.8. (a) Weight-mantle length and (b) weight-total length relationships from *Octopus huttoni* collected from 2004 to 2006 (n=250) and from 2017 to 2018 (n=88).

The *b* values for the relationship between ML and TL for both the Otago (*b*=0.4939, t-test, p=8.188 x 10^{-12}, df=46) and Bluff (*b*=0.4953, t-test, p=2.783 x 10^{-15}, df=40) populations were significantly different from 1 indicating negative allometric growth (Fig. 2.9).

The relationship between ML and TL for both year-datasets both had *b* values that were significantly lower than 1 (2004-2006, *b*=0.685, t-test, p=3.327 x 10^{-8}, df=247) (2017-2018, *b*=0.5548, t-test, p=4.243 x 10^{-13}, df=87) indicating negative allometric growth for both where ML grows faster than TL (Fig. 2.10).

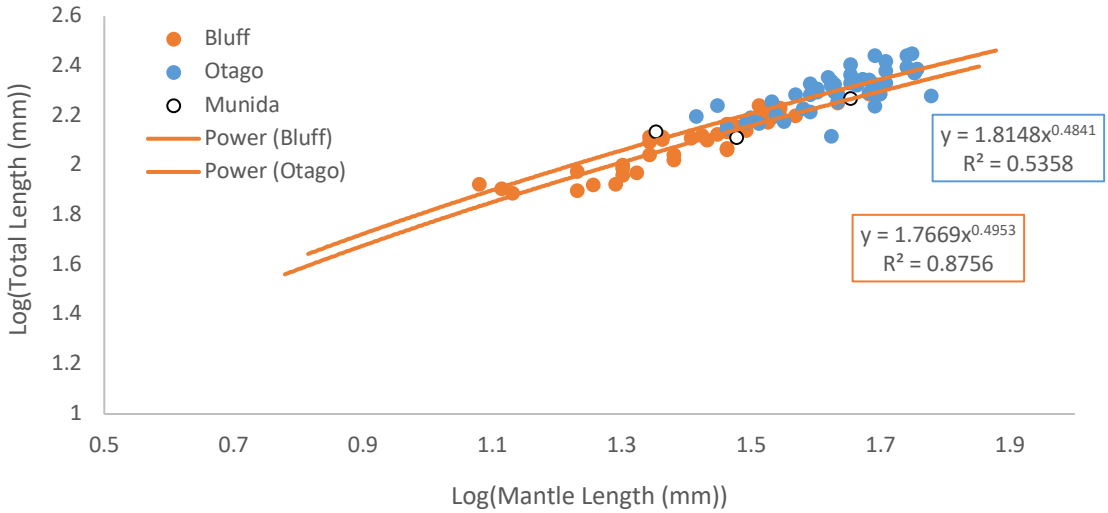

Figure 2.9. Mantle length-total length with log transformation between *Octopus huttoni* from Otago (n=44), Bluff (n=41), and Munida (n=3) fit with power curves.

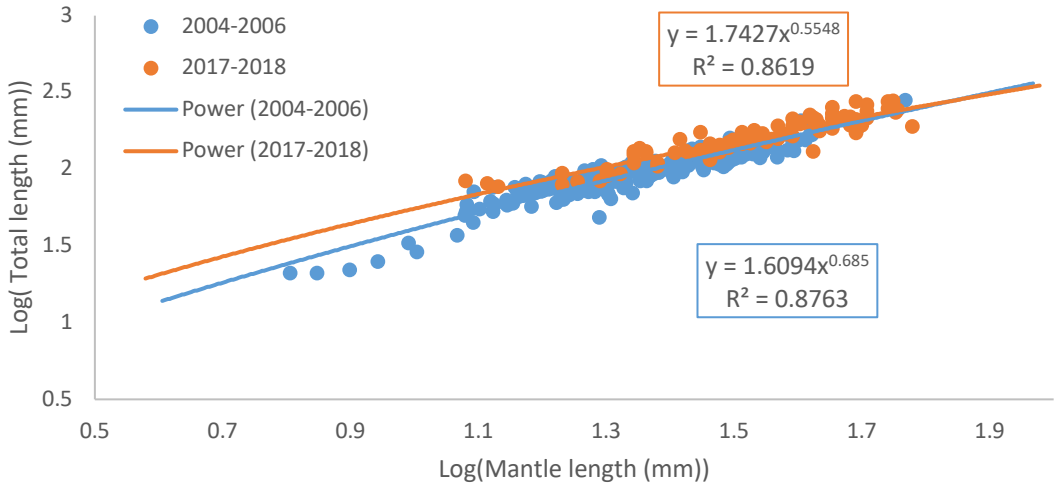

Figure 2.10. Mantle length-total length relationship comparison between *Octopus huttoni* collected from 2004 to 2006 (n=250) and from 2017 to 2018 (n=88) fit with power curves.

2.3.4 Size of first maturity and sexual dimorphism

The smallest male recorded (ML: 21 mm, WW: 2.572 g) was from Bluff and had a small hectocotylus starting to form on the third, right arm (Fig. 2.3). The end of the hectocotylus was thicker and lacking suction cups whereas a normal tentacle is much thinner at the tip and has suction cups all the way down. On average, females and males were the same approximate size, but the majority of larger individuals were identified as males (Fig. 2.11). It should be noted that 44% of females measured were spent.

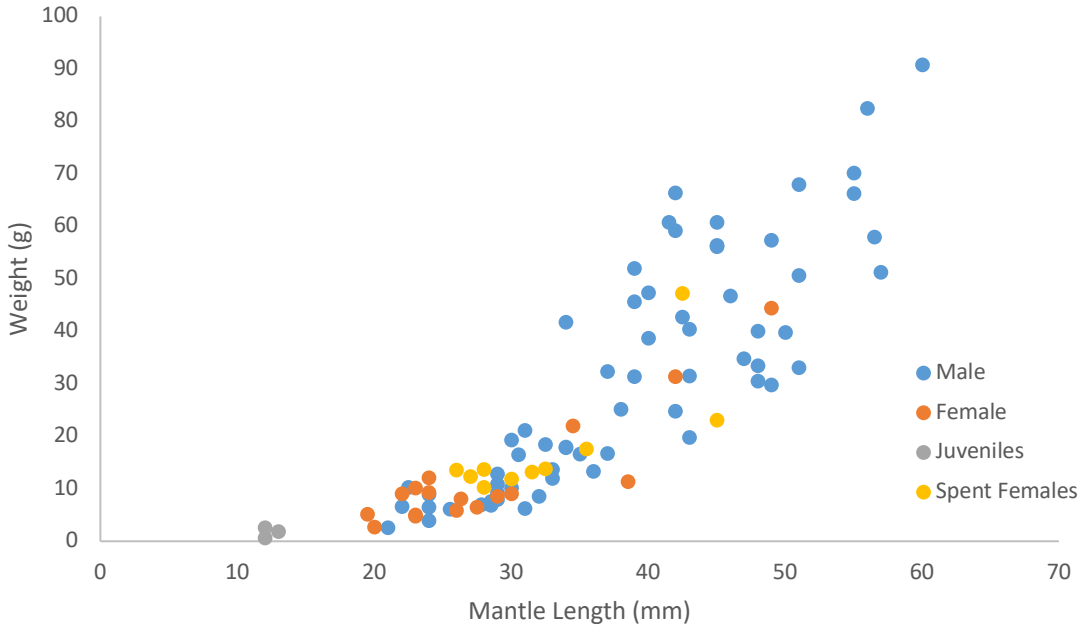

Figure 2.11. Wet weight (g) against mantle length (mm) for male (n=64), female (n=27), juvenile (n=3) and spent female *Octopus huttoni* (n=21) from Otago and Bluff. Of the females, 44.4% were spent (already laid eggs and brooding).

2.4 Discussion

This study provided data on the size, condition, and growth variation between three populations of *O. huttoni* in Southern New Zealand and data collected from 2017-2018 and 2004-2006.

2.4.1 Age of maturity in Octopus huttoni

The smallest mature male (ML: 21 mm, WW: 2.572 g) may represent the size at first maturity because the hectocotylus was so small but identifiable. To be sure, more samples around this size should be carefully sexed by looking at sexual organs and inspecting the third, right arm. This will confirm if animals smaller than this size are indeed juveniles and not just small females.

Previous studies have found that *M. maorum, O. vulgaris,* as well as other species of octopus have asynchronous sexual maturity so that they may reach maturity at

any time throughout the year (Grubert & Wadley, 2000; Silva *et al.*, 2002; Cuccu *et al.*, 2013). This is seen as male *O. vulgaris* and *O. maya* reach maturity before females (Hernando *et al.*, 2009; Silva *et al.*, 2002; Cuccu *et al.*, 2013; Perales-Raya *et al.*, 2014b). This strategy is beneficial because unmated females are rare as they can store sperm for up to 10 months so males maturing quickly therefore increase their chances of encountering an unmated female in their life span (Grubert & Wadley, 2000).

Size at maturity in *O. vulgaris* has been recorded but two largely different estimations were made. Cuccu *et al.* (2013) recorded that a mature size for males is 320 g and 520 g for females. This is much lower than Silva *et al.'s* (2002) estimations of 671 g for males and 2023 g for females. Cuccu *et al*. (2013) attributes these differences to the different habitats as their study was done in the western Mediterranean basin and Silva *et al.* (2002) was in the Gulf of Cadiz, south of Portugal. These data imply that size of maturity in the same species can vary between populations. If this small male is an indication of the size of maturity in *O. huttoni*, then it suggests that this species could have rapid sexual maturity like most other octopods.

2.4.2 Male vs female size variation

Many of the larger individuals were male, but 44.4% of the females were measured after brooding eggs and starving for up to 4 months. Octopus females are known to shrink during the brooding process as they are starving (Moltschaniwskyj, 1995; O'Dor & Wells, 1978; Anderson *et al.*, 2001) and may have been the same size as males before spawning but shrunk while brooding. Therefore, males were identified as larger than the females.

O. bimaculoides, Octopus pallidus, and *O. vulgaris*, also have males which are larger than females, so this pattern is not unusual in octopods (Forsythe & Hanlon, 1988; Sanchez *et al.*, 1998; Leporati *et al.*, 2008b). Most larger animals may have been male because larger females are most likely brooding eggs and therefore won't crawl into traps as frequently as males. Future studies should measure animals

upon capture to get a more accurate wild-caught size estimate as many animals in the present study were measured after maintaining in captivity for several weeks.

2.4.3 Distribution and size variation of Octopus huttoni

O. huttoni found offshore in the Foveaux Strait were significantly smaller than those caught in the Otago Harbour. This could suggest that those found in the harbour are older and therefore larger. Smaller *O. huttoni* may be found in offshore waters because they spend approximately 40-70 days floating/weakly swimming in the water column before settling to the benthos (Carrasco, 2014). Stranks (1996) and Otero *et al.* (2009) suggested that merobenthic octopus with planktonic young such as *O. huttoni* could be transported offshore as planktonic larvae by inshore coastal currents and upwelling. This idea is logical as the main advantage of having a planktonic stage is to increase distribution (Stranks, 1996; Otero *et al.*, 2009). When comparing spatial genetic structure in *M. maorum*, Higgins *et al.* (2013) found that patterns appeared to be related to oceanographic circulation systems. This indicates that larval dispersal in this species is dependent on currents (Higgins *et al.*, 2013). The same phenomenon could also be occurring in *O. huttoni* as it is also a merobenthic species with a similar distribution as *M. maorum.*

A further hypothesis is that after being carried offshore and settling to the benthos in the open ocean, individuals may migrate back to coastal waters where they reproduce. González *et al*. (2011) and Mayo-Hernández *et al.* (2013) suggest that *O. vulgaris* females move inshore to spawn, but whether this is the case for males is unknown. Munida individuals were either statistically similar to Otago or Bluff indicating that they represent an intermediate population where they are smaller than those in the Harbour, but larger than those from Foveaux Strait. This could be due to the temperature, depth, or benthic environment in Munida compared to Foveaux Strait.

An alternative hypothesis is that *O. huttoni* in inshore waters grow quicker and to a larger size than offshore animals. This could be due to availability of nutrients and

food, temperature at depth, and the type of substrate (Chapter 1). Substrate in the Foveaux Strait and offshore of Otago is composed of biogenic structures such as bryozoan and molluscs patch reefs in which the octopus hide (Cranfield *et al.*, 2004; Mather, 1982). The substrate in the Otago Harbour is soft sediment, which is increasingly muddy as dredging of the harbour continues to widen the channel for large ships (Single *et al.*, 2010). A separation of different sized octopus may occur in these habitats due to these variables that influence food availability and growth. To investigate this, ages of octopus between the inshore and offshore locations would need to be compared to see if there are indeed younger octopus offshore or if they are just smaller. If younger animals are found offshore, an ontogenetic shift in habitat may occur and if they are the same age but smaller, they may represent two completely distinct populations

2.4.4 Allometric growth variation

From the power growth curves that were fit to the data, it is apparent that growth never reaches an asymptote or maximum size, which is consistent with other studies (López-Rocha *et al.*, 2012; Herwig *et al.*, 2012; Forsythe, 1984; Forsythe & Hanlon, 1988; Leporati *et al.*, 2007). The exponents increased drastically when ML was replaced with TL in the weight-length relationship indicating that it might not be appropriate to use TL because the shape of the animal is too complex (arms). It is more representative of the data if ML is used as length because it is essentially a sphere/oval and can be compared with weight. In this discussion ML is used instead of TL to represent length.

The weight-length relationship between locations (Otago and Bluff) both produced *b* exponents below 3 indicating negative allometric growth in which length grew faster than weight. But the *b* exponent for Bluff individuals was not significantly different from 3 indicating that it is more isometric than Otago individuals. When the two curves were compared, the octopus from Otago appeared to grow larger in WW indicating that they might be older as well. Age will be estimated in the next chapter, to test this theory. If this is true, juveniles would be displaying isometric

growth and adults negative allometric growth in which ML grows more rapidly than WW.

Negative allometry in weight-length relationships is common in octopods such as *O. bimaculatus* (López-Rocha *et al.*, 2012), *Bathypolypus sponsalis, Eledone moschata, Octopus salutii, O. vulgaris*, and *Scaeurgus unicirrhus* (Merella *et al.*, 1997). But so is positive allometry in other species such as *Eledone cirrhosa* and *Pteroctopus tetracirrhus* (Merella *et al.*, 1997). A variety of abiotic and biotic factors can affect these growth results such as age, temperature, and food availability, but growth also varies between individuals (Van Heukelem, 1976; Hanlon, 1983; Forsythe & Van Heukelem, 1987; Forsythe, 1984). There is extreme variation in growth even in individuals reared the exact same (Van Heukelem, 1976; Hanlon, 1983; Forsythe & Van Heukelem, 1987; Forsythe, 1984) so further studies are needed to fully understand why these patterns exist.

The weight-length relationship between individuals of both year-datasets produced *b* exponents above 3, but the t-test showed that neither were significantly different from 3. Although allometry classifies both as isometric, from the exponents, we can see that the 2004-2006 dataset is more positively allometric than the 2017-2018 dataset. This indicates that the weights of individuals caught from 2004-2006 were increasing faster in relation to ML than those caught more recently. Reasoning behind this may be that in the recent years, there has been less food available in the Foveaux Strait for these midget octopus. From 2012 to 2016, the estimated abundance of oysters in Foveaux Strait declined more than 50% from 473.9 million in 2012 , to 211.3 million in 2014, declined further to 55.4 million in 2015, and then slightly increased to 88.8 million in 2016 (Michael & Forman, 2016). This decline can either be attributed to the outbreak of *Bonamia*, a waterborne parasite that infects and kills oysters, the oyster fishery, the destruction of habitat due to dredging, or a combination of all of the above (Michael & Forman, 2015). If *O. huttoni* is feeding on oysters which are currently being harvested, it is a possibility that this fishery is removing a valuable resource for midget octopus. Future research should determine if these octopus utilize oysters as a food resource and how they will be affected by continued dredging.

In the length-length comparisons between locations and year-datasets, all allometric curves displayed negative allometry and are significantly different from 1. This indicates that ML grew quicker than TL for all individuals. Again, Otago individuals were larger than Bluff individuals indicating that they might be older.

Variation in allometric growth between location and between individuals caught in different decades might also be attributed to temperature differences. Ricker (1979) suggests that animals found in the colder limits of their geographical range tend to grow to a larger final size than those in the warmer limits. This was seen in the cuttlefish *Sepia sp*. where a smaller final size was reached by those reared in higher temperatures than those in lower temperatures (Forsythe & Van Heukelem, 1987). It is a possibility that this could occur in this species, but Otago surface water is only about 1°C on average colder than that in Foveaux Strait (Appendix A1). Also, individuals in Foveaux Strait are living at greater depths than those found in the harbour and it can be predicted that temperature is colder at these deeper depths. If this theory is correct, those from Bluff would have a larger final size unless the opposite is true as in *O. vulgaris* where a larger final size is attained when reared at warmer temperatures (Smale & Buchan, 1981). This could give insight on why the Otago individuals are larger, but currently, no assumptions can be made as temperature at depth data from the site of capture would be needed. In the past 50 years, sea surface temperature has increased in Southern New Zealand at a rate of 0.10°C decade^{-1} due to the warming of subtropical waters (Shears & Bowen, 2017). This increase in temperature could explain the size increase of 2017-2018 caught octopus compared to 2004-2006 caught octopus as the warming water may be causing different growth rates.

Octopus such as *O. vulgaris* are known to grow at faster rates when temperature is higher and to grow faster, more food needs to be consumed (Smale & Buchan, 1981; Forsythe & Van Heukelem, 1987). This was seen in the current study. Although octopus sizes were not measured throughout maintenance in captivity, there was an increase in feeding during warmer periods. During the hot months of the summer (December-March), octopus ate noticeably more crabs than during the colder months (May-June). There was an unexpected decrease in feeding

during January- February, which may be due to the infestation of parasites at this time (parasite data found in Appendix B). These infected octopus would stop eating for a few days up to a couple of weeks before dying. It was later discovered that they were infected with parasites. This was also during the marine heat wave in which temperatures were much higher than normal (Appendix C1) which could have cause higher infection rates and in turn, higher death rates.

2.4.5 Parasites in Octopus huttoni

O. huttoni caught from the Foveaux Strait in 2004-2006 were significantly smaller than those caught in 2017-2018 except for HW. HW may not have been significantly different between datasets as the head is not as plastic as other parts of the body such as the arms. This overall difference in size could be due to the increased prevalence of parasites infecting *O. huttoni* as none were found during 2004-2006, but four different types were found in 2017-2018 (Appendix B).

2.4.6 Conclusions

In conclusion Otago individuals were possibly larger than Bluff individuals because they were older or were growing to larger sizes because of differences in habitat. Individuals caught more recently in 2017-2018 were larger than those caught in 2004-2006 possibly because of the increase in temperatures. But 2004-2006 individuals had more positive allometric WW-ML relationship, which could be due to a loss of condition in the more parasitized 2017-2018 individuals. These size comparisons are a good starting point and an easy way to visualize morphometric data, but just size alone is not an indicator of age especially in wild-caught animals, which is why age estimates must be made (Forsythe & Van Heukelem, 1987). With age data, we can see how size changes over their lifespan and between locations.

Chapter 3: Stylet and beak growth increments

3.1 Introduction

3.1.1 Aging and population biology

Age and growth information is crucial for understanding the life history of an organism which is essential when managing and conserving populations (Doubleday *et al.*, 2006; Herwig *et al.*, 2012; Cuccu *et al.*, 2013; Lourenço *et al.*, 2015). Age estimates are essential to quantify the growth rates, size at maturity, population age structure, recruitment and mortality rates (Doubleday *et al.*, 2006). Despite the increase in commercial catches and the ecological importance of cephalopods as a bridge between benthic and pelagic food chains, there is limited information on cephalopod growth patterns (Doubleday *et al.*, 2006; Doubleday, 2009; Doubleday *et al.*, 2016; Rodhouse *et al.*, 2014). In the case of *Octopus huttoni*, although it is not a commercially targeted species, it can be indirectly affected by human activities such as trawling and dredging. By understanding the life history of this species, it is possible to understand how it is being affected, and thus take actions to prevent negative impacts on the *O. huttoni* populations.

3.1.2 Growth increments

The idea of aging a marine animal using its hard parts, such bones and scales, was first observed by Aristotle (ca. 340 BC) who stated in his *Historia Animalium* (ca. 340 B.C.) that "the age of a scaly fish may be told by the size and hardness of its scales" (Thompson, 1910: Book VIII, Section 20, cited in Jackson, 2007). This idea was then ignored until Antoni van Leeuwenhoek, one of the first acknowledged microscopists, observed circular lines on the scales of eels (Leeuwenhoek, 1685, cited in Jackson, 2007). He then concluded that the scale deposited one circular line every year (Leeuwenhoek, 1685, cited in Jackson, 2007) and later, he correctly determined that the growth pattern is similar to trees in which the darker areas indicate a season of slowed growth (Leeuwenhoek 1798, cited in Jackson, 2007). It was not until 1898 when C. Hoffbauer studied this in fish and concluded that the rings in scales could be used for aging. Soon after Hoffbauer's publication,

Johannes Reibisch published a paper on using growth rings within the otolith of the plaice, *Pleuronectes platessa*, instead of scales in order to age fish (Reibisch, 1899, cited in Jackson, 2007). Subsequently, studies have been completed on a range of different species of freshwater vertebrates such as the three-spotted tilapia (Booth *et al.*, 1995), bluegills (Kowalewski *et al.*, 2012), and the redband trout (Schill *et al.*, 2010) and marine vertebrates such as the pacific cod (Roberson *et al.*, 2005) and warty oreo (Stewart *et al.*, 1995) to validate otoliths as an aging method. Fish aging was incorporated into fisheries assessments in the early 1900's (Jackson, 2007). As this method became more popular, researchers (Williamson 1918 cited in Jackson, 2007) questioned the validity of the techniques that Reibisch used in his original work, however the method is now widely used to gather aging data for fisheries assessments (Jackson, 2007). Fish scales and otoliths are now extensively used for aging fish and these age estimates, along with knowledge about size at maturity, are employed in managing fisheries around the world.

Statoliths are the cephalopod equivalent of the fish otolith as they are also calcareous, located in the head and aid in balance and orientation of the animal (Budelmann, 1980; Leporati *et al.*, 2008b; Arkhipkin *et al.*, 2018). Clark (1966) first reported that squid statoliths have growth increments similar to those of fish otoliths and subsequently, statoliths and other hard parts, such as squid pens have been used to estimate age in cephalopods (Rodhouse & Hatfield, 1990). Unfortunately, the statoliths in octopods are crumbly because they have randomly arranged statoconia (calciferous granules) and do not lay down increments as in squid, so different forms of estimating age are needed (Arkhipkin *et al.*, 2018; Robinson & Hartwick, 1986; Doubleday *et al.*, 2006; Leporati *et al.*, 2008b; Barratt & Allcock, 2010).

3.1.3 Using stylets for aging

More recently, stylets have been used for estimating age in octopods (Doubleday *et al.*, 2006). Stylets in incirrate octopods (species without fins), such as *O. huttoni*, are a pair of thin translucent, cartilage-like rods, which are embedded in the

mantle muscle tissue behind the two branchial hearts (Wells, 1978; Bizikov, 2004) (Fig. 3.1a). In cirrate octopods, or finned octopus, their paired stylets are fused making a U-shape and are dubbed gladii (Fig. 3.1b) (Bizikov, 2004). A gladius differs from a stylet in that the gladius supports the rudimentary fins whereas the function of stylets in incirrate octopus is unknown.

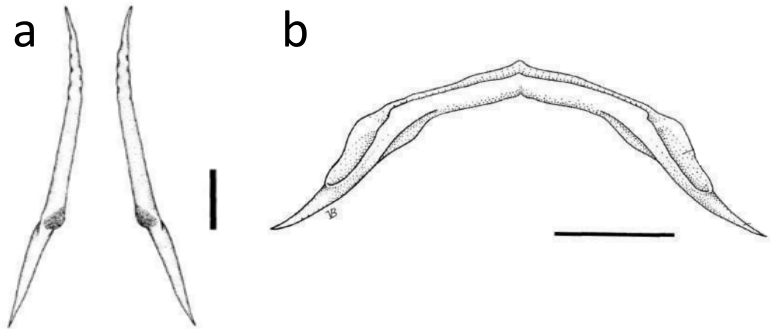

Figure 3.1. (a) Stylets of the incirrate octopus, *Enteroctopus dofleini*. (b) Gladii of the cirrate octopus, *Opisthoteuthis californiana*. (Adapted from Bizikov, 2004). Scale bars = 1 cm.

Sousa Reis and Fernandes (2002) first reported counts of growth increments from the stylet in *Octopus vulgaris* and suggested that they could be used for aging. They compared the number of increments to morphometric data such as total length (TL) and mantle length (ML), but could not conclude that these lines represented age (Sousa Reis & Fernandes, 2002). Doubleday *et al*. (2006) later carried out an aging experiment to count the number of growth rings in stylets of laboratory reared *Octopus pallidus* of known age. This resulted in a discussion of the use of stylets to estimate age of wild-caught octopus. Since then, many studies have used stains such as calcein or tetracycline to create marks in the stylets of live octopus in order to validate the periodicity of growth increments (Table 3.1). This is done by creating two or more marks with known time intervals between the stains and comparing the number of growth increments between the marks to the actual number of days in the time period (Lipinski, 1986; Jackson, 1989; Villanueva, 2000; Hermosilla *et al.*, 2010). Daily deposition of growth rings has been validated in *O. vulgaris*, *O. pallidus*, *Octopus maya*, and *Octopus tetricus* (Table 3.1 and Table 3.2), but this cannot be assumed for all species. This is because each species lives in a different habitat with different life histories which may potentially affect the periodicity in which growth increments are deposited in stylets.

Table 3.1. Studies that validated cephalopod growth periodicity with a stain.

Species	Hard part	Stain	Method	Reference
Sepia officinalis	Statolith	Hydrochloride tetracycline	Injection in arm	(Bettencourt and Guerra, 2001)
Idiosepius pygmaeus	Statolith	Tetracycline	Immersion in water bath (500 mg $2L^{-1}$) for 2 hours	(Jackson, 1989)
Loligo vulgaris (paralarvae)	Statolith	Tetracycline	Immersion in water bath (250 mg L^{-1}) for two hours	(Villanueva, 2000)
Octopus vulgaris	Stylet	Tetracycline	Injection in arm (120 mg kg^{-1} body weight)	(Hermosilla *et al.*, 2010)
Alloteuthis subulata	Statolith	Oxytetracycline (OTC) and chlortetracycline (CTC)	Injection intramuscularly of the mantle (100 mg OTC 1 mL^{-1} seawater, 10 mg CTC 2 mL^{-1} distilled water)	(Lipinski, 1986)
Loliolus noctiluca	Statolith	Tetracycline	Ambient exposure (250 mg L^{-1})	(Jackson, 1990b)
Loligo chinensis	Statolith	Tetracycline	Injection (6 mg mL^{-1})	(Jackson, 1990b)
Sepioteuthis lessoniana	Statolith	Tetracycline and calcine	Immersion in water bath (100 mg L^{-1} of calcein for 1.5 hours, 250 mg L^{-1} of tetracycline for 2 hours)	(Jackson, 1990a)
Illex illecebrosus	Statolith	Tetracycline	Given with food/force fed	(Dawe, 1985)
Todarodes Pacificus	Statolith	Tetracycline	Given with food	(Nakamura and Sakurai, 1991)
Onychoteuthis borealijaponica	Statolith	Tetracycline	Ambient exposure	(Bigelow, 1994)

Recently, stylet weight has been investigated as an indirect octopus aging method in that stylet weight increases with age (Leporati & Hart, 2015). By using stylet weight, it is not necessary to prepare stylet sections and therefore risk damaging the sample.

Table 3.2. Species of octopus that have been aged using Stylet Increment Analysis (SIA), Beak Increment Analysis (BIA), and lipofuscin quantification.

Species	Aging method	Deposition validated?	Reference
Octopus vulgaris	SIA	Yes (tetracycline staining and known-aged hatchlings)	(Barratt and Allcock, 2010; Hermosilla *et al.*, 2010; Lourenço *et al.*, 2015)
	BIA		(Raya and Hernández-González, 1998; Hernández-López and Castro-Hernández, 2001; Perales-Raya *et al.*, 2010; Cuccu *et al.*, 2013; Perales-Raya, Almansa, *et al.*, 2014; Perales-Raya, Jurado-Ruzafa, *et al.*, 2014)
Octopus pallidus	SIA	Yes (from known-aged animals)	(Doubleday *et al.*, 2006; Leporati *et al.,* 2008)
	Lipofuscin		(Doubleday and Semmens, 2011)
Octopus maya	SIA	Yes	(Rodríguez-Domínguez *et al.*, 2013)
	BIA	Yes (from known-aged animals)	(Rodríguez-Domínguez *et al.*, 2013; Villegas-Bárcenas *et al.*, 2014)
Octopus cyanea	SIA	No	(Herwig *et al.*, 2012)
Octopus tetricus	SIA	No	(Ramos *et al.*, 2014)
	SIA and stylet weight	Yes (calcine staining)	(Leporati and Hart, 2015)
Macroctopus maorum	SIA	No	(Doubleday *et al.*, 2011)
Bathypolypus sponsalis	SIA	No	(Barratt and Allcock, 2010)
Megaleledone setebos	SIA	No	(Barratt and Allcock, 2010)
Eledone cirrhosa	SIA	No	(Regueira *et al.,* 2015)

3.1.4 Using beaks for aging

More recently, beaks have been used as a method for aging octopus as long as their age could be validated by known-aged individuals (Table 3.2) (Hernández-López *et al.*, 2001; Perales-Raya *et al.*, 2010, 2014a, 2014b; Villegas-Bárcenas *et al.*, 2014). Cephalopod beaks are comprised of a chitin-protein complex which grow from secretions of a single layer of tall columnar cells called beccublasts (Dilly & Nixon, 1976; Hunt & Nixon, 1981; Uyeno & Kier, 2005). These beccublasts create the growth lines which can be seen on the surface of the beak. Beak increments were first described by Clarke (1965) who found them in the squid *Moroteuthis ingens* on the lateral walls of the lower beak (Fig 3.2). Clark did not validate these lines as daily, but he noted that the micro-rings appeared to be dependent on an abiotic factor such as temperature or food availability (Clarke, 1965). After this, Raya and Hernández-González (1998) used the beak to estimate age and compare the number of increments in *O. vulgaris* beaks to morphometries such as body mass and sex. Although they did not validate absolute age using the growth lines, they suggested that they were most likely laid down daily (Raya & Hernández-González, 1998).

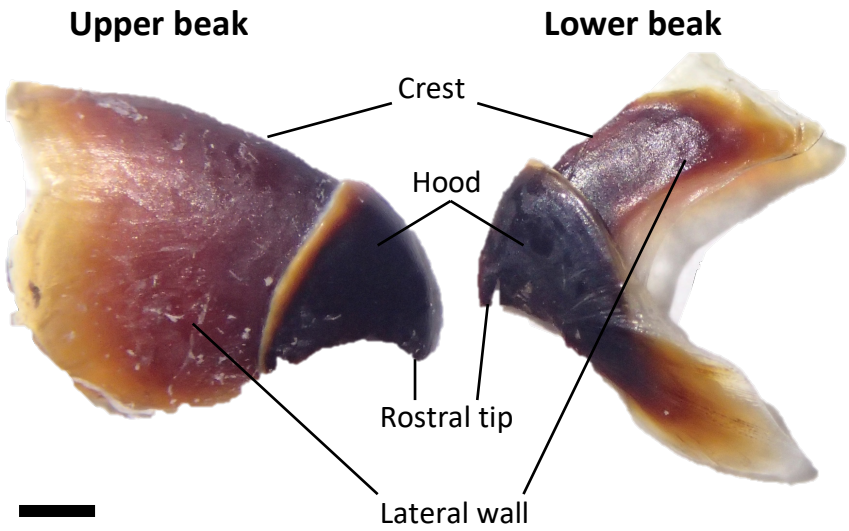

Figure 3.2. Diagram of the upper and lower beak of *Octopus huttoni*. Location of crest, hood, rostral tip, and lateral wall are indicated. Scale bar = 1 mm.

There are two approaches to increment counting in beaks; rostrum sagittal sections (RSS) and lateral wall surfaces (LWS) which can be applied to the upper and lower halves of the beak. The LWS method involves cutting the beak (usually the upper beak) in half and counting the lines on the lateral wall (Fig. 3.3) (Clarke, 1965; Hernández-López *et al.*, 2001; Perales-Raya *et al.*, 2010). The RSS method involves embedding the beak in resin and then taking sagittal parallel sections (0.5-0.8 mm) with a diamond saw and mounting on a slide (Fig. 3.4) (Raya & Hernández-González, 1998). This method has been improved by cutting the section first and then embedding in resin (Perales-Raya *et al.*, 2010). The sections are then ground with a fine grade sandpaper (1200 grit), polished with 3 and 1 µm diamond paste, and sometimes etched with 8.5% di-sodium ethylenediaminetetraacetate (EDTA) solution or 37% HCl (Raya & Hernández-González, 1998; Perales-Raya *et al.*, 2010). Upper and lower beaks can be used for RSS, but the upper beak has proven to have more increments suggesting that it might be more accurate (Raya & Hernández-González, 1998; Hernández-López *et al.*, 2001).

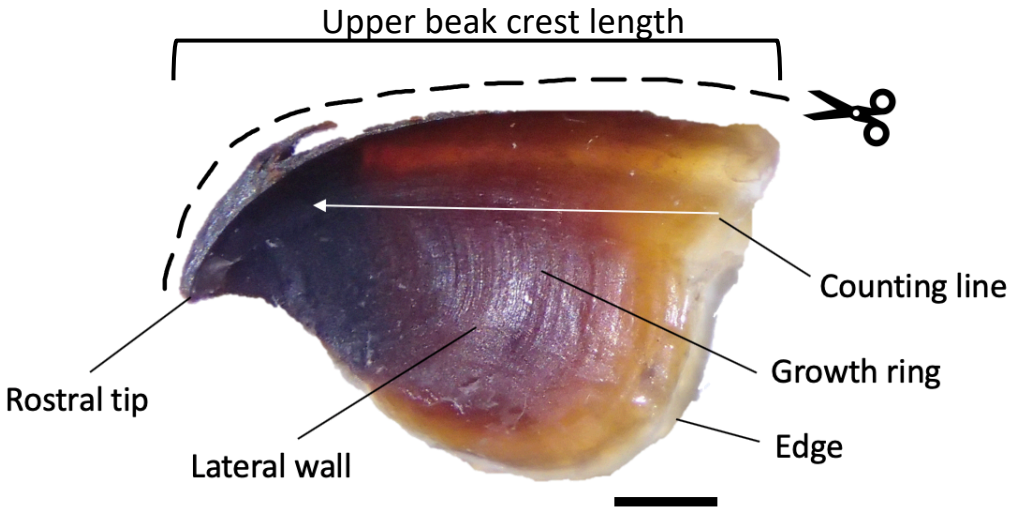

Figure 3.3. Photo of *Octopus huttoni* upper beak with cutting line indicated by the dotted line, counting line indicated by the white arrow, and growth ring, rostral tip, lateral wall, and beak edge indicated by black lines. Upper beak crest length is indicated by the horizontal bracket. Scale bar = 1 mm.

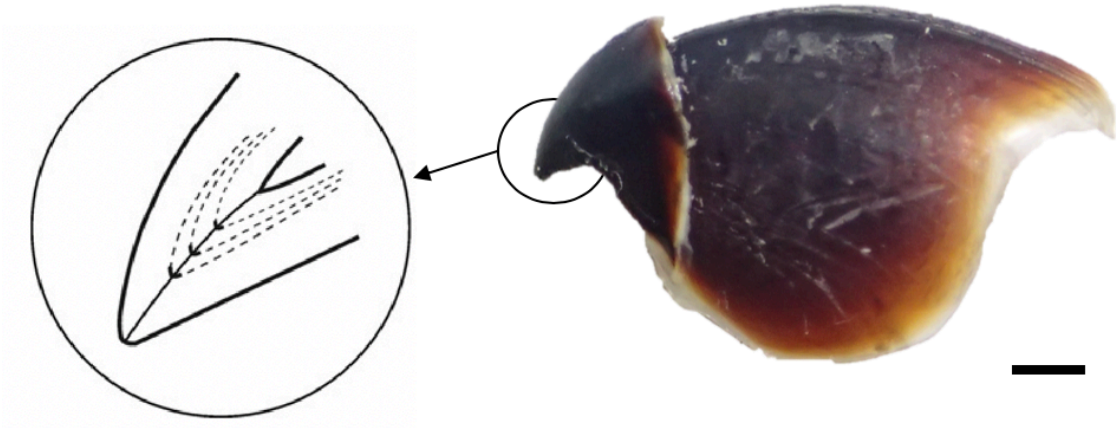

Figure 3.4. Diagram of the area of increment counting and increments (inset) using rostrum sagittal sections in the upper beak of *Octopus huttoni* (Adapted from Perales-Raya *et al.*, 2010). Scale bar = 1 mm.

Often, beak length is used as an indirect method for aging or estimating size of the octopus when evaluating prey items of higher tropic level animals such as birds (Lalas & Mcconnell, 2012; Fea *et al.*, 1999; Lalas, 2009). Like stylet weight, this is an easy method to estimate size and relative age of the specimen.

3.1.5 Modelling growth

Cephalopods are known for being fast-growing animals with indeterminate growth. Indeterminate growth can be modelled by the von Bertalanffy growth function ($y=L_{\infty}(1-e^{-Kx})$), which assumes that food is abundant and that growth approaches an asymptote or maximum size (Moltschaniwskyj & Carter, 2010; von Bertalanffy, 1938). This function has been used in many fish studies to model growth and even some cephalopod studies have shown that the von Bertalanffy curve can be fitted to octopus growth (Guerra, 1979; Forsythe & Van Heukelem, 1987). But since the von Bertalanffy curve assumes that growth slows as it reaches the maximum asymptotic size, this model may not be appropriate for octopus as they are not known to reach an asymptotic size (Guerra, 1979; Forsythe & Van Heukelem, 1987). Another model that may fit octopus growth more appropriately is the Richards function ($y=L_{\infty}/(1+e(-K*(x-t0)))$). This is a four-parameter variation of the three-parameter von Bertalanffy growth function, which is logistic in shape and more flexible than the exponential von Bertalanffy (Richards, 1959; Ricker, 1979). Both models have the parameters; maximum size (L_{∞}), growth rate

(K), and age (x), but the Richard's curve also has a point of inflection (t0) because it is a sinusoidal curve.

3.1.6 Aims

The present chapter aimed to determine the best aging method for wild-caught *O. huttoni*, estimate age to compare populations (Fig. 2.1), and model size at age growth using the von Bertalanffy and Richard's growth curves. Aging methods involving the stylet and beak were used in wild-caught midget octopus to determine the applicability of each method; (1) beak increment analysis, (2) upper beak crest length, (3) stylet increment analysis, and (4) stylet weight.

3.2 Methods

Methods describing octopus capture, animal husbandry, and euthanasia are given in Chapter 2. The beak and both stylets were extracted from each individual and placed straight into 70% ethanol until further analysis.

3.2.1 Octopus huttoni *beak growth rings and length*

In this study, ages of wild-caught octopus were estimated using the LWS method because LWS has been reported to be closer to chronological age than the RSS method (Perales-Raya *et al.*, 2010, 2014a). Beaks of *O. huttoni* were cut longitudinally in half (Fig. 3.3) with scissors, cleaned lightly with fresh water and a scalpel or plastic pipette to scrub off any residual tissue (beaks with tissue still attached made it difficult to clearly read the increments). One half of the beak was fixed to a microscope slide with Crystalbond™ (Fig. 3.5). Care was taken to ensure that the Crystalbond™ was not heated to its boiling point to minimize bubbles and was cooled slightly so that the beak would not curl when it came into contact with the hot resin. Tweezers were used to gently flatten the beak so that more surface area could be viewed through the microscope. Careful treatment was needed as the small beaks were found to be brittle.

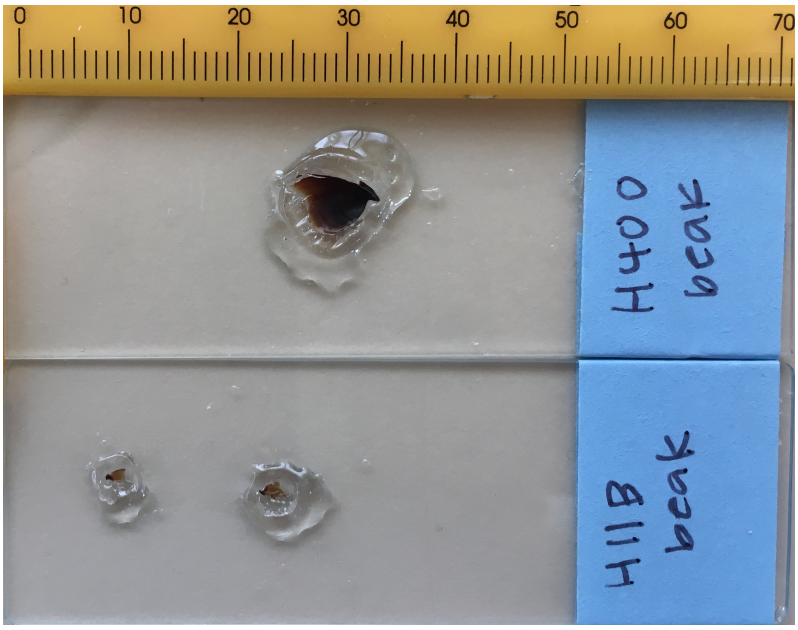

Figure 3.5. Comparison of the largest (top) and smallest (bottom) *Octopus huttoni* beaks mounted on a slide with thermoplastic cement Crystalbond™. Scale bar in mm.

The increments on each beak's lateral wall were hand counted three times non-consecutively by a single reader. A subset of 10 samples from Otago and 10 samples from Bluff were counted by an experienced second reader (Dr. Jean McKinnon) for a total of six counts for those 20 samples. Counts were made under a phase-contrast microscope with the aid of a camera Lucida drawing arm at 100x magnification. If an increment ended, view was shifted vertically to count all increments visible (staggered growth phenomenon). The staggered growth phenomenon refers to the incremental disappearance or split into a larger number of increments (Lipinski, 1993), as seen in squid statoliths and fish otoliths (Wild and Foreman, 1989; Lipinski, 1993). For each reader, if the counts were more than 10% different, another reading was taken to ensure a total of three counts with <10% difference. The counts were then averaged and the coefficient of variation (CV) was calculated. The samples that were counted by both readers were then compared using a scatter plot with a linear trendline fitted (points sitting on a slope of 1 being identical counts by both readers). A total of 109 beaks were counted; 43 from Otago Harbour (Otago), seven from Otago Shelf (Munida), and 59 from Foveaux Strait (Bluff). Beak counts and death date were then used to back-calculate date of first increment formation assuming daily deposition of growth rings.

Crest length of all upper beaks was measured to the nearest 0.01 mm to determine if beak length was correlated with the age and size of the individual. In JMP pro (version 11.0) Shapiro-Wilk tests were used to check for normality and one-way ANOVAs were run to test if number of beak increments and beak crest length were significantly different between the three sites (Otago, Bluff, and Munida). Tukey post-hoc tests were run to further investigate the differences.

In addition, 53 beak age estimates from a 2004-2006 dataset (Fiona Higgins, unpublished data) were compared to the present study.

3.2.2 Increment validation with paralarvae beaks

An attempt to age known-aged paralarvae using their beaks was made. Paralarvae preserved in 70% ethanol were dissected using 27-gauge needles to remove beaks which were then mounted on a microscope slide with glycerol jelly and a cover slip. Beaks had to be extracted in water as they were very brittle from long exposures to ethanol. Beaks from freshly deceased paralarvae were the easiest to extract as they have not been exposed to ethanol. Because the beaks were so delicate, they were left whole and butterflied with the inner portion face up instead of halved. The inner lateral wall of the upper beak just below the teeth (Fig. 3.6) was then observed for increments and photographed at 400x and 1000x magnification.

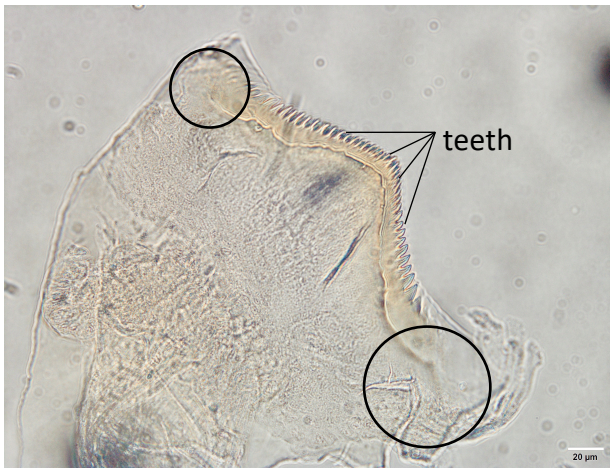

Figure 3.6. Upper beak of 12-day old *Octopus huttoni* paralarvae. The circle indicates the region in which increments are formed and examples of teeth are identified. Scale bar = 20 µm.

3.2.3 Octopus huttoni *stylet growth rings and weight*

M. maorum specimens were readily available as they wash up naturally on shore after spawning during the winter, eliminating capture time. These specimens were used to practice dissections and preparation of stylet sections. Many versions of the permanent stylet embedding method described by Barratt and Allcock (2010), were trialled in the present study using *M. maorum* stylets but produced preparations with unclear increments. Therefore, a new method was used where stylets were embedded whole instead of sectioning first. For mounting, moulds were made with a glass base, double sided tape, and a cylindrical section (Fig. 3.7). The stylets were placed vertically on the tape with the rostrum flat on the base and the post-rostral zone pointing straight up. If the stylet was broken at the bend, the post-rostral zone was stood on the end where the bend would be and supported by a small glass tube (Fig. 3.7). Resin (Wests system 105) was mixed with the hardener (Wests system 206) and then cured in a 60°C oven for 3 minutes to force out any air bubbles. The resin was then carefully poured into the moulds as to not knock over any stylet pieces and then cured in the 60°C oven for a further 2 hours. After cooling, the cylindrical moulds were removed from the base and resin blocks were removed from mould. The blocks were ground down on the base side using a coarse mechanical sander (120 grit) until a surface from the region of increment analysis was visible (Fig. 3.8), and further sanded on a fine sander (240 grit). That side was then mounted on a microscope slide and cut to ~0.5 mm with a diamond saw. The section was polished by hand with 1200 grit sandpaper and then on 6 µm and 3 µm diamond pads until the section was ~100 µm in thickness as 80 µm was found to be too thin and layers would be ground away.

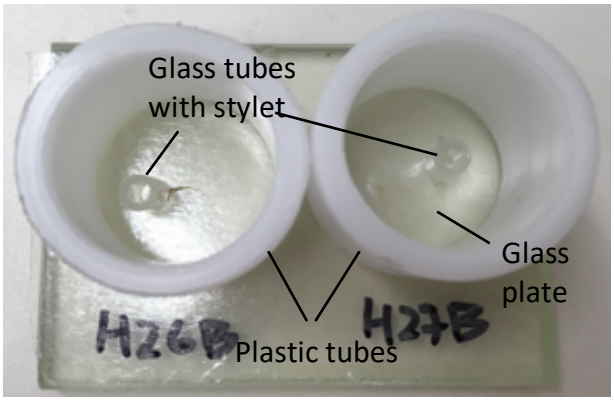

Figure 3.7. Stylet embedding mould created with a glass plate, double sided tape, small glass tubes, and larger plastic tubes.

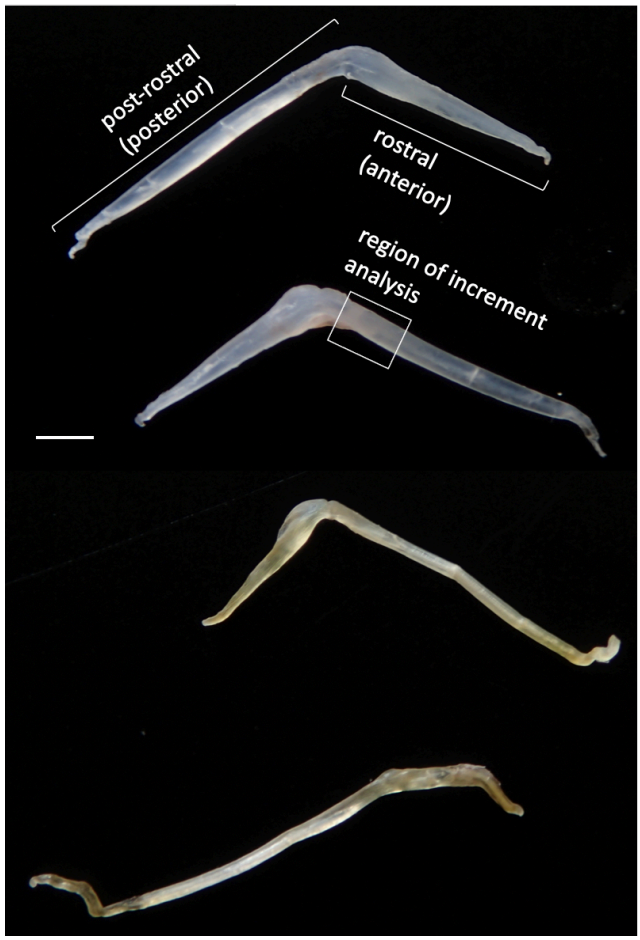

Figure 3.8. Photos of typical octopus stylets (*Octopus huttoni)* post extraction showing the variation in shape and thickness. Scale bar = 1 mm.

Increments on stylets were counted in a similar fashion as the beaks, but from micrographs as opposed to direct counting. Micrographs were taken under a compound microscope (Olympus BX51) fixed with a camera (Olympus XC50) under 1000x magnification with oil immersion. One reader counted all visible increments three times non-consecutively from the best micrograph of each sample. As with the beaks, if there was more than a 10% difference, additional independent counts were made to get <10% difference. Stylets from 47 individuals from Otago, six from Munida, and 28 from Bluff were prepared and counted. Eight stylets from Otago, one from Munida, and 31 from Bluff were not analysed due to poor preparation.

The weight of intact stylets was determined from 29 individuals from Otago Harbour, two from Munida, and 21 from Bluff to the nearest 0.01 mg to establish

stylet weight with age. Only whole stylets were weighed as it could not be determined if pieces were missing from broken stylets. Stylets were weighed individually and if both stylets from an individual were whole, an average was taken. In JMP pro (version 11.0) Shapiro-Wilk tests were used to check for normality of the stylet increment and weight data. If not normally distributed, non-parametric tests were used. Chi-squared tests were done to determine if the number of stylet increments and stylet weight were significantly different between locations. Tukey post-hoc tests were then performed to further investigate the differences.

3.2.4 Daily increment validation

Six live octopus of varying size from Bluff and 17 from Otago were stained with tetracycline, an antibiotic, which acts as a florescent stain. For this, 3 g of tetracycline were dissolved in 250 mL of seawater, heated to 30°C and stirred with a stir bar for 10 minutes. The tetracycline mixture was then strained through a 240 µm mesh net and added to an enclosed tank with 11.75 L of seawater (final tetracycline concentration: 250 mg L^{-1}). Octopus were placed in the tank where they stayed for two hours as per Jackson (1990) and Villanueva (2000). After, they were placed in a clearing bucket with 15 L of fresh seawater for another two hours before placing them back in their original 55 L tanks. After 60 days of the initial staining, all except three individuals from Otago were stained again in the same manner. Those three individuals were not stained a second time because they either died or were euthanized due to poor condition. Thirty days after the second staining, the animals were euthanized using a lethal dose of AQUI-S and then frozen for later dissection.

A second method of staining was applied because the water bath method proved to produce stylets without obvious fluorescent lines. In this second method, nine animals from Otago were anesthetised using cold water (0-2°C), injected, and put back in home tanks to recover. Care was taken to ensure that the animals were under anaesthesia for as short as time possible to avoid permanent damage (under five minutes). A stock solution (5 mg mL^{-1} to 10 mg mL^{-1}) made of hydrochloride

tetracycline (HTC) and autoclaved seawater (pH 5) was made immediately prior to injection to prevent the solution from oxidising. A total of 0.12 mg HTC was administered per gram of body weight by injection with a 27-gauge needle at the base of the thickest arm (Hermosilla *et al.*, 2010). A concentration of 5 mg mL^{-1} was used to start and then it was realized that some individuals were heavier and would need more than 1 mL of solution. Therefore, a more concentrated stock was made to avoid injecting unnecessary amounts of liquid. Eight individuals were stained twice within a 20-day period between the two injections and then euthanised by cold-water (-0.5-2°C)10 days after the second injection.

After staining, stylets were dissected out, placed in a centrifuge tube containing 70% ethanol, and the tube was wrapped in aluminium foil to prevent oxidation of the tetracycline. The stylets were then prepared in a similar fashion as the non-stained stylets. Care was taken to reduce exposure to light by working in a dimly lit room. Transverse sections, <0.5 mm in length were cut from the post-rostral zone near the bend and fixed on a microscope slide with thermoplastic cement (Crystalbond™) or glycerol jelly and a cover slip. The Crystalbond™ sections were then ground with 400, 800, then 1200 grit sandpaper and polished with 5 µm aluminium oxide paste with a cloth until transparent. Sections were excited under a UV filter (excitation: 300-360 nm) using a mercury lamp (Olympus U-RFL-T) and micrographs were taken under 1000x magnification. Fluorescent micrographs were compared to white light micrographs so marks could be matched with the corresponding growth line. Total growth rings were then counted three times non-consecutively, as with non-stained stylets. Because the entire stylet from the centre to the last stain fluoresced and no noticeable fluorescent lines were found, the number of growth rings from the edge of fluorescence to the edge of the stylet were counted. These counts represent the number of rings laid down between the most recent stain and death.

3.2.5 Fitting models

The best hard part for counting increments was determined by fitting both sets of data to each other and then fitting a 1:1 trendline. The method that was

determined to be the most accurate was then used to fit growth models. In these age-size models, ML was used instead of TL because it is more representative of the animal's size as arms can break, shrink, and stretch. A von Bertalanffy growth model and a Richard's curve were fitted to the size at age data using Microsoft Excel (version 16.20) and the r^2 values and the predicted coefficients were used to determine the best fitting model.

3.3 Results

3.3.1 Beak increments

When cleaned thoroughly, increments on the beak were easy to read (Fig. 3.9). More light was needed to see increments on the rostrum near the tip of the beak because of the thickness of this area (Fig. 3.9).

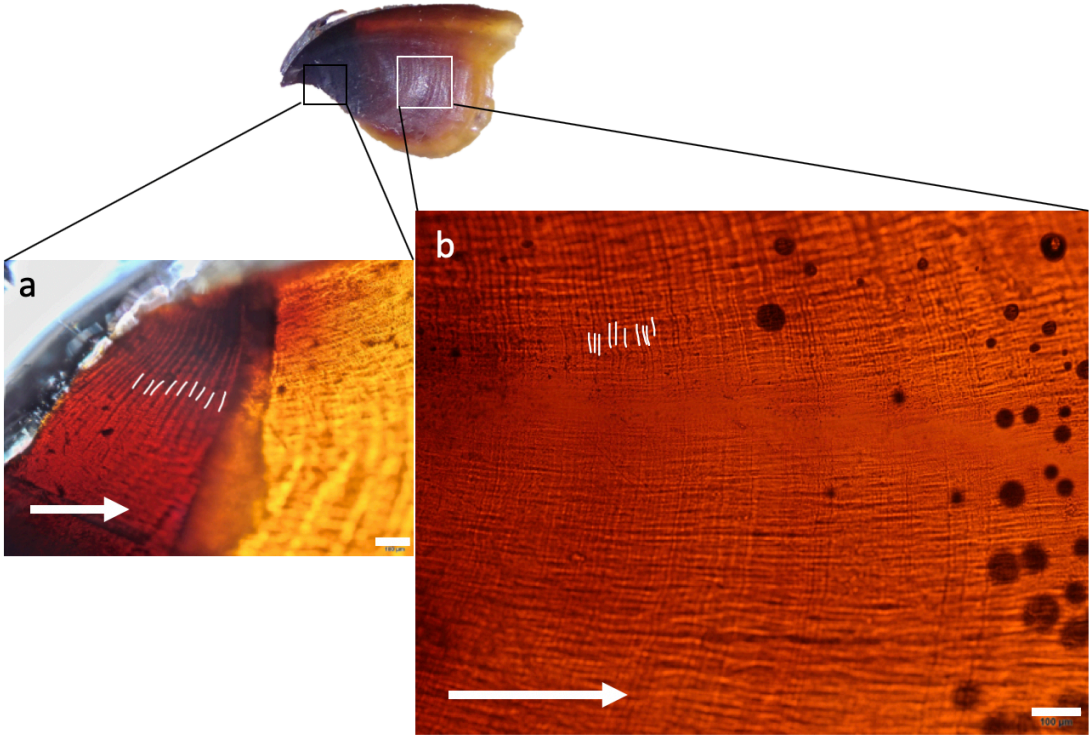

Figure 3.9. Increments on *Octopus huttoni* (a) rostrum and (b) lateral wall of beaks. The arrow indicates direction of growth from oldest to newest increments. Examples of increments are overlaid with white Scale bars = 100 μm.

The precision comparison between the two readers fit a linear trend (Fig. 3.10). Those samples which deviated further from the trend line were those which were not cleaned thoroughly and therefore harder to read.

The back calculation showed that individuals formed their first beak increment all year-round with a peak in September and October (Spring) (Fig. 3.11).

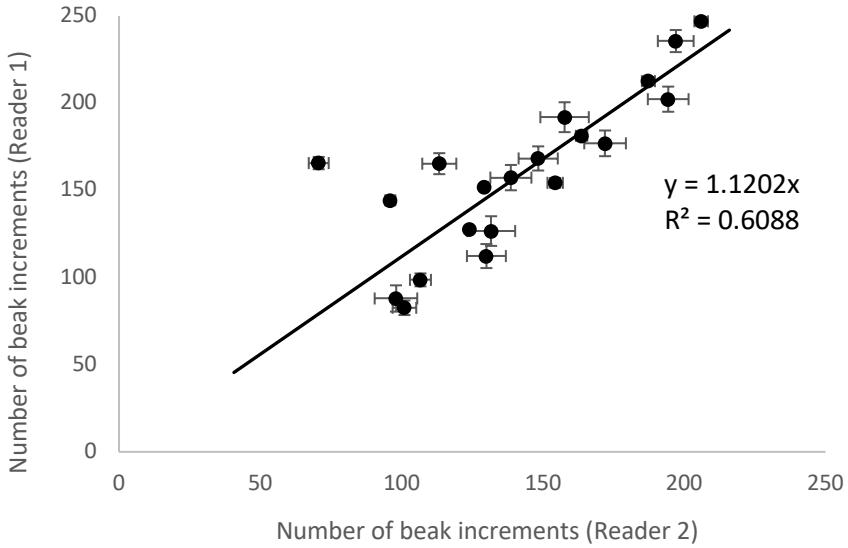

Figure 3.10. Comparison between mean beak count estimates (n=20) of *Octopus huttoni* from two readers with a linear trendline forced through 0 and standard error bars.

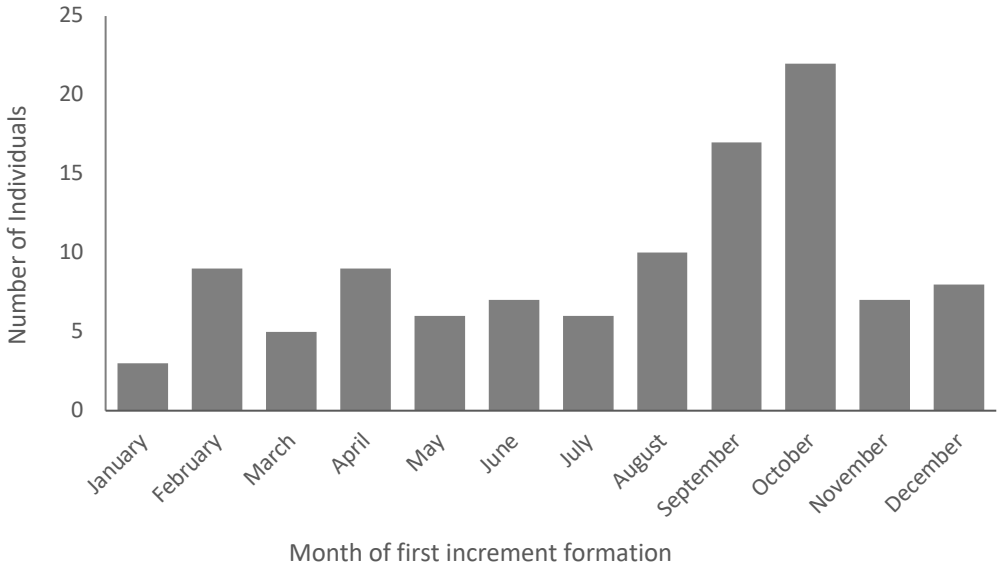

Figure. 3.11. Back calculation of when *Octopus huttoni* individuals formed the first beak increment using age estimated from beaks and death date assuming that one increment equals one day.

The number of beak increments (x) correlated with mantle length (y) and wet weight (y) fit power curve trends well (Fig. 3.12). Individuals from Otago were older (108-250 increments, mean=179) than those from Bluff (28-215 increments, mean=123) with individuals from Munida in-between (86-205, mean=156), but on the younger side of the Otago range (Fig. 3.13). There was a significant difference between the number of increments for the three sites (ANOVA, $F_{(2, 108)} = 75.08$, $p<0.0001$). The Tukey post-hoc test showed that beak counts of Otago and Bluff individuals were significantly different and beak counts of Munida individuals were not significantly different than the other two locations (Fig. 3.13).

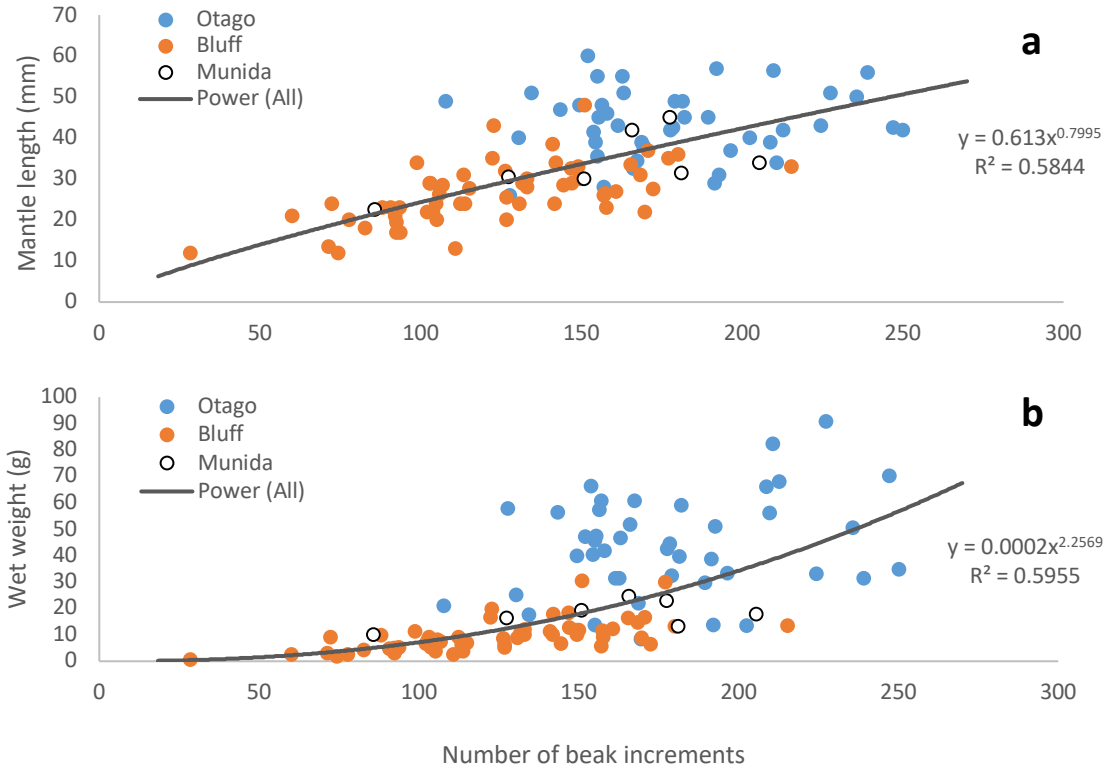

Figure 3.12. Relationship between beak increment count (age) and (a) mantle length (mm) or (b) wet weight (g) from three populations of *Octopus huttoni* from Otago (n=43), Bluff (n=59), and Munida (n=7) fit with power curves.

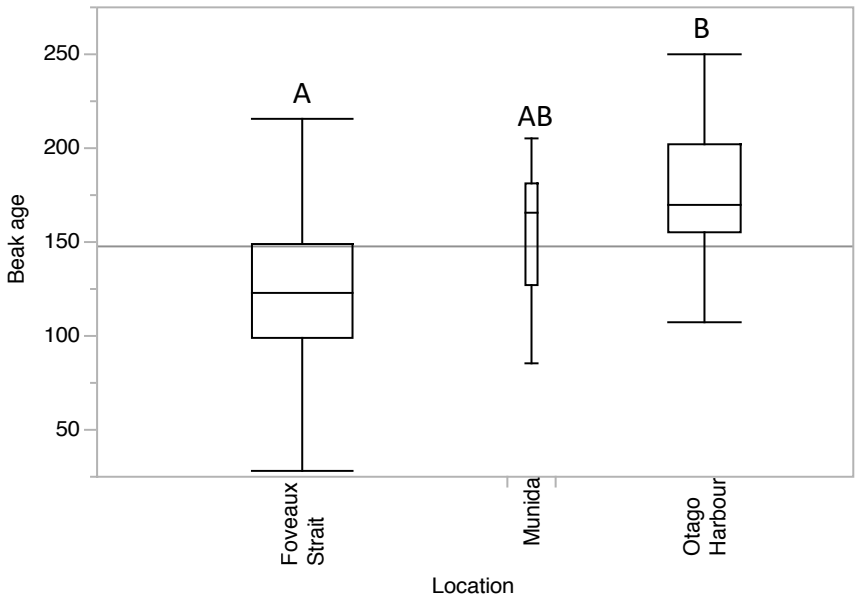

Figure 3.13. Box plot of the estimated beak ages (days) from *Octopus huttoni* from Foveaux Strait (n=59), Otago Harbour (n=43), and offshore Otago continental shelf (Munida)(n=7). The horizontal line in the middle of each box is the median, the upper line is the upper quartile, the lower line is the lower quartile, the upper bar is the maximum value, and the lower bar is the minimum value for that site. The grey horizontal line through the plot represents the median of all of the data. Those indicated by different letters are significantly different ($p<0.05$).

When beak counts from the 2004-2006 data were added to the current counts, the data mixed in well with the Bluff samples and younger Otago samples from the present study (ANOVA, $F_{(1, 111)} = 1.261$, $p=0.264$) (Fig 3.14).

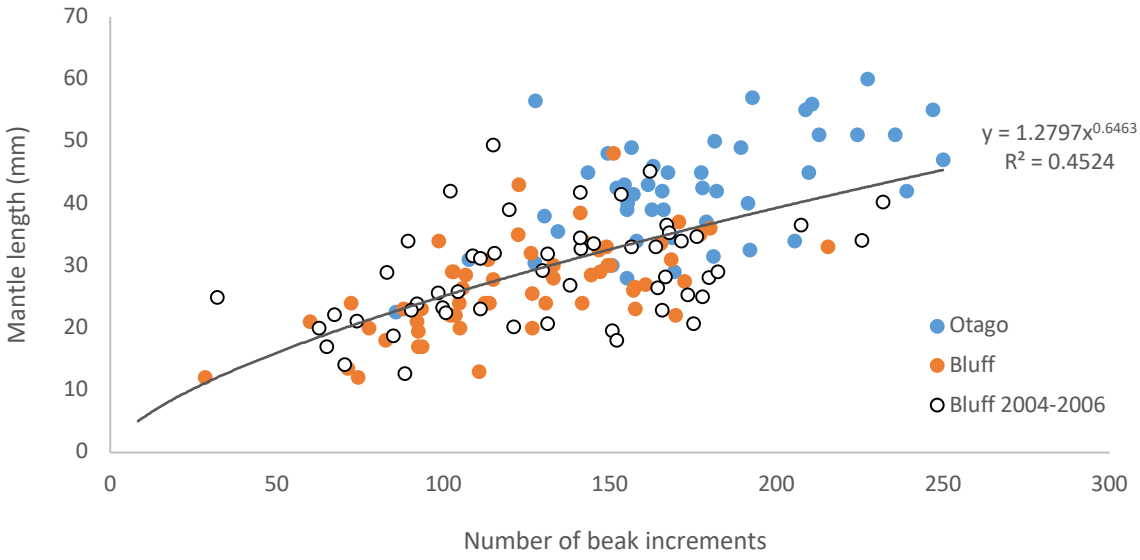

Figure 3.14. Change in mantle length (mm) with age estimated from beak increments from *Octopus huttoni* sourced from Otago (n=50), Bluff (n=59), and Bluff from 2004-2006 (n=53) fit with a power curve.

Upper beak crest length (y) (mm) increased exponentially with mantle length (x) (mm) ($y=3.089e^{0.020x}$, $r^2=0.76$) and beak estimate (x) ($y=3.098e^{0.005x}$, $r^2=0.60$) (Fig. 3.15). Otago individuals had larger beaks (5.72-10.13 mm, mean=7.91), than those from Munida (5.79-8.52 mm, mean=6.91) and those from Foveaux Strait who had the smallest (3-7.12 mm, mean=5.07) (Fig. 3.16). There was a significant difference in beak length between the three locations (ANOVA, $F_{(2, 108)} = 140.46$, $p<0.0001$) and the Tukey post-hoc further described the locations as significantly different (Fig. 3.16).

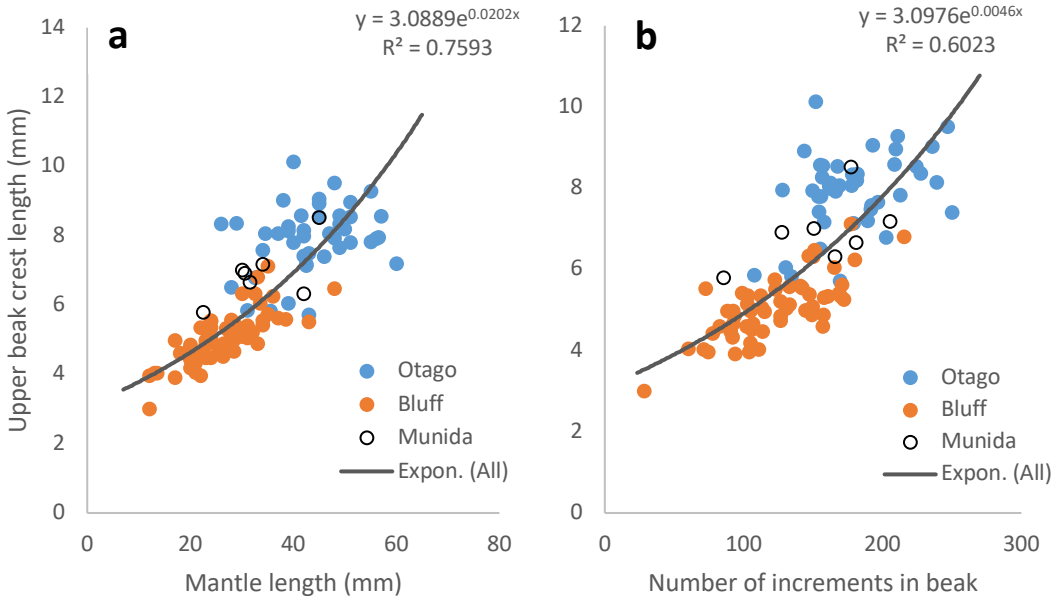

Figure 3.15. *Octopus huttoni* upper beak crest length (mm) compared to the (a) mantle length (mm) and (b) number of increments in the beak of individual from Otago (n=43), Bluff (n=59), and Munida (n=7). Fit with exponential curves.

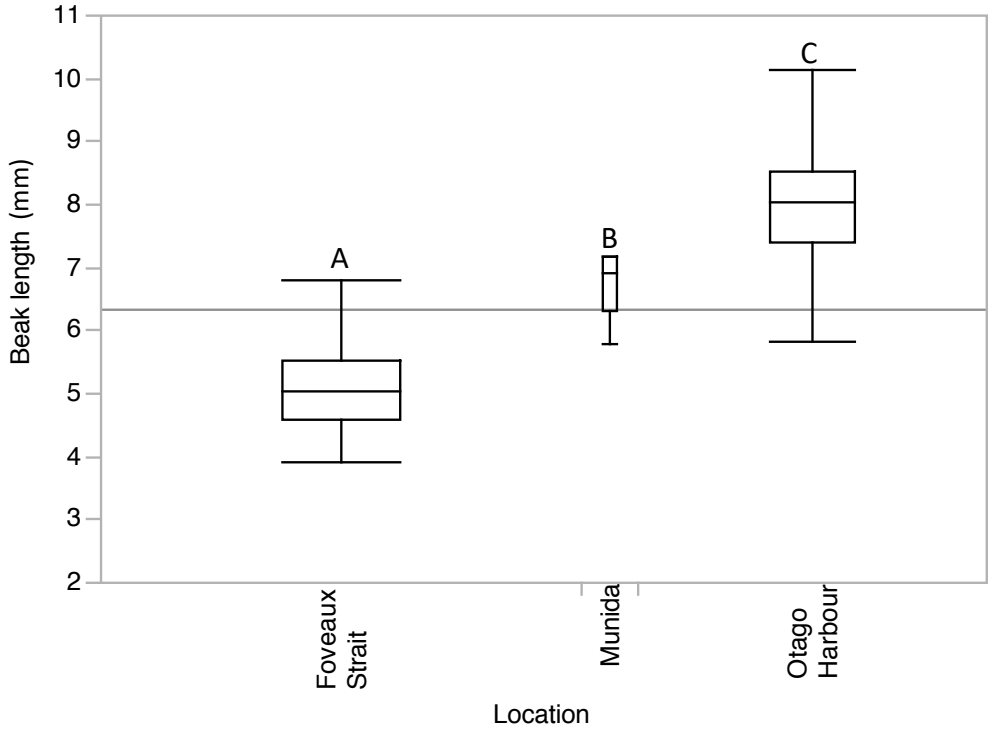

Figure 3.16. Box plot of upper beak crest length (mm) between *Octopus huttoni* from Foveaux Strait (n=59), Otago Harbour (n=43), and the Otago shelf (Munida)(n=7). The horizontal line in the middle of each box is the median, the upper line is the upper quartile, the lower line is the lower quartile, the upper bar is the maximum value, and the lower bar is the minimum value for that site. The grey horizontal line through the plot represents the median of all of the data. Those indicated by different letters are significantly different ($p<0.05$).

3.3.2 Increment validation using paralarvae beaks

No visible increments were found on paralarval beaks, but a series of fine lines were seen fanning out from one point (Fig 3.17). These were not concluded to be growth increments as they were found in 12-day old individuals and the number of lines exceeded 20.

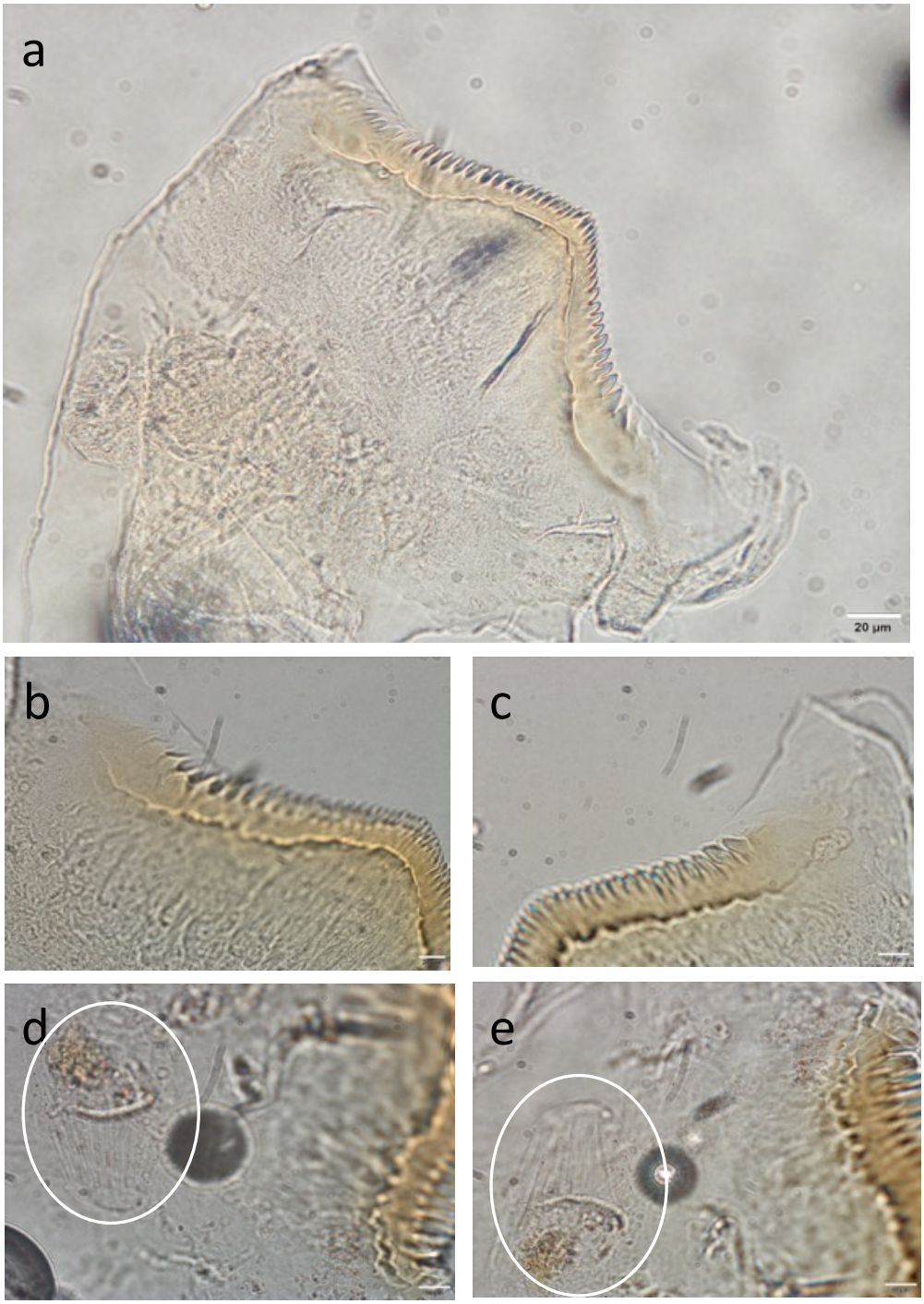

Figure 3.17. Images of *Octopus huttoni* paralarvae beaks with close ups of regions of incrementation. (a) scale bar = 20 µm. (b), (c), (d), (e) scale bar = 10 µm. White circles indicating areas where lines are seen.

3.3.3 Stylet increments

The stylet preparation methods from Barratt and Allcock (2010) produced stylet sections which were slightly deformed due to peeling of increment layers (Fig. 3.18). This made it difficult to count because of the gaps in layers. Therefore, *O.*

huttoni stylets were embedded whole, cut with the diamond saw and ground. This produced sections which were whole without gaps (Fig. 3.19). When prepared carefully and at the right thickness, stylets were easy to read. When prepared with too much heat or friction, darkening or gaps between layers would occur; hindering the visibility of increments. The resulting stylet counts were lower and more variable than beak counts because of the increased difficulty in readability.

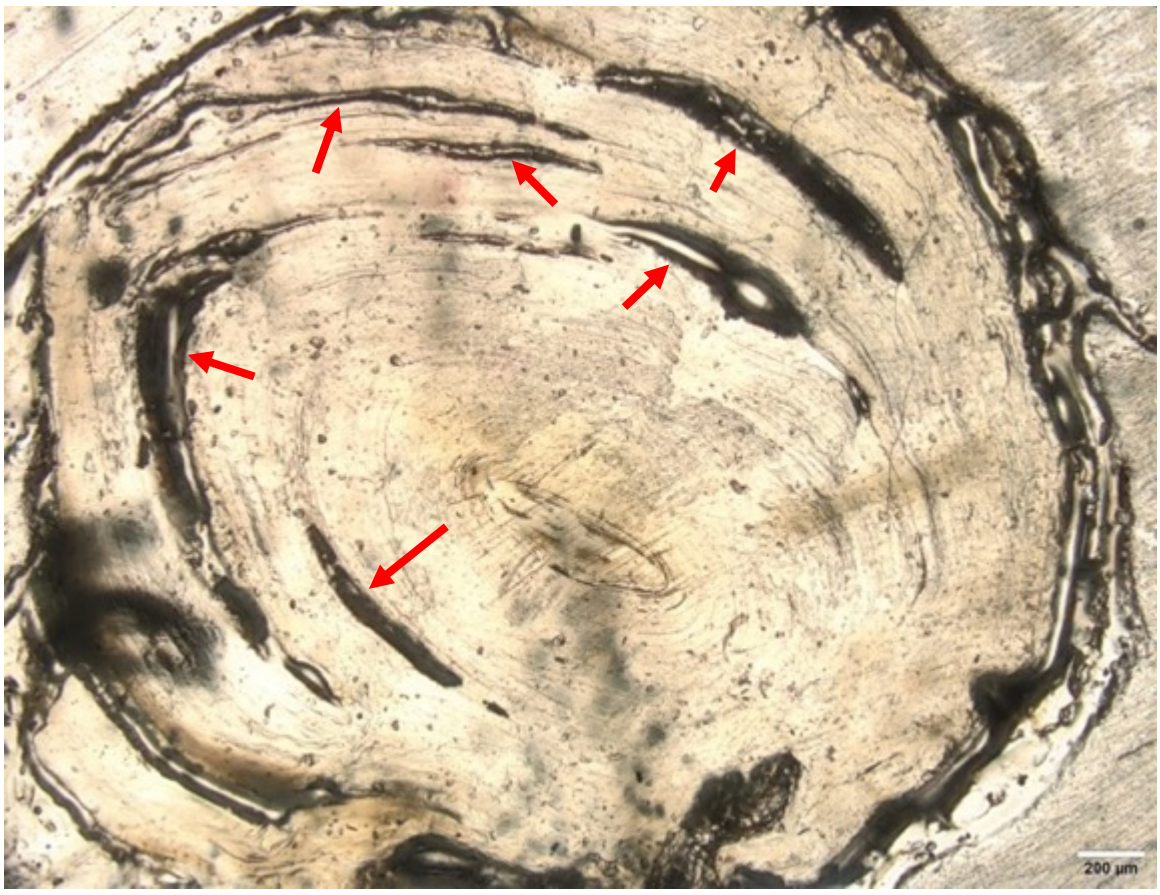

Figure 3.18. *Macroctopus maorum* stylet section prepared using the methods of Barratt and Allcock (2010). The red arrows indicate areas where the layers are peeling off from each other creating gaps. Scale bar = 200 µm.

The shape of *O. huttoni* stylets are similar to those in other species in the Octopus genus, with a shorter anterior side and longer posterior side (Fig. 3.8). Often if the stylet was too thin such as those from younger individuals, it would break in half during extraction. When prepared correctly and at the correct thickness, stylet increments were relatively easy to read except for the centre or nucleus (Fig. 3.19). Because the stylet is very soft and increment layers are extremely thin, any grit on top or under the stylet hindered readability greatly.

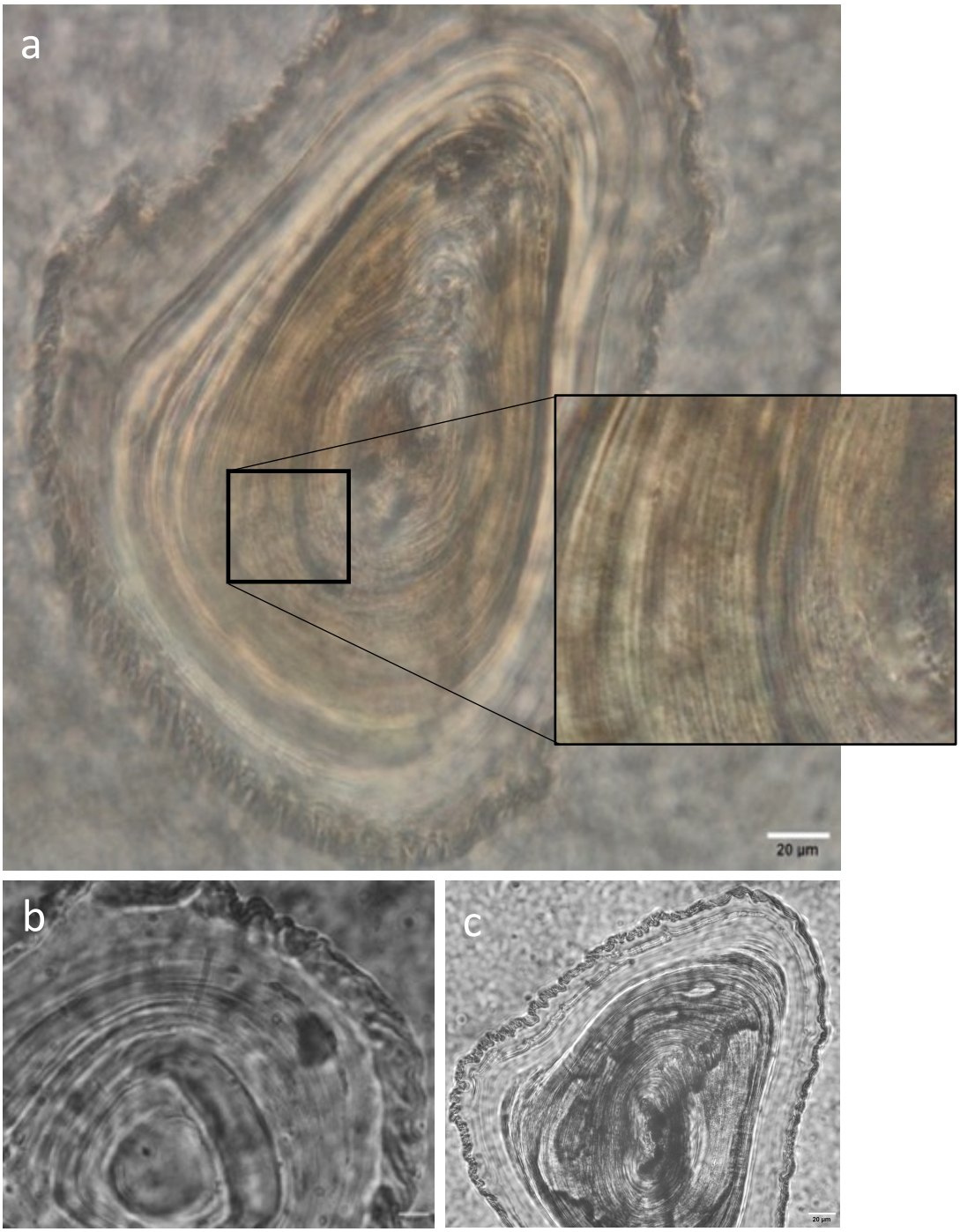

Figure 3.19. Examples of *Octopus huttoni* stylet photos with a zoomed in portion to better show increments. (a) scale bar = 20 µm. (b) and (c) scale bar = 10 µm.

Similar to the beak age estimates, stylet age estimates increased allometrically when compared to mantle length and weight, (mantle length: $y=-5.782x^{0.399}$, $r^2=0.17$, weight: $y=0.029x^{1.449}$, $r^2=0.30$) (Fig. 3.20). Stylet age estimates also showed that individuals from Otago were older (67-222 increments, mean=122) than those from Bluff (53-131 increments, mean=82) and individuals from Munida

were intermediate (51-116 increments, mean=90) (Fig. 3.21). There was a significant difference in stylet age estimate between locations (χ^2 (2, N = 81) = 32.41, p<0.0001). The Tukey post-hoc further indicated that stylet estimates from Otago individuals were significantly different from Bluff and Munida individuals (Fig. 3.21).

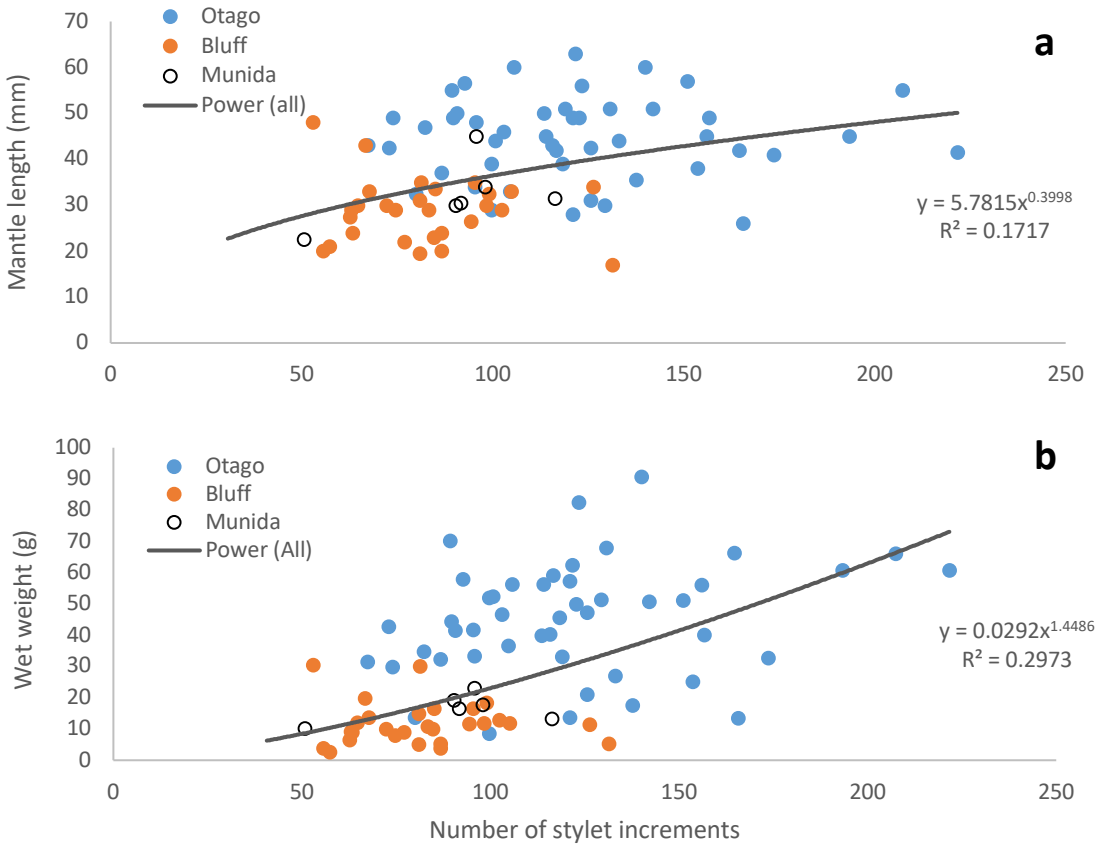

Figure 3.20. Change in (a) mantle length (mm) and (b) weight (g) as the number of increments in the stylet increases from *Octopus huttoni* from Otago (n=47), Bluff (n=28), and Munida (n=6). Fit with power curves.

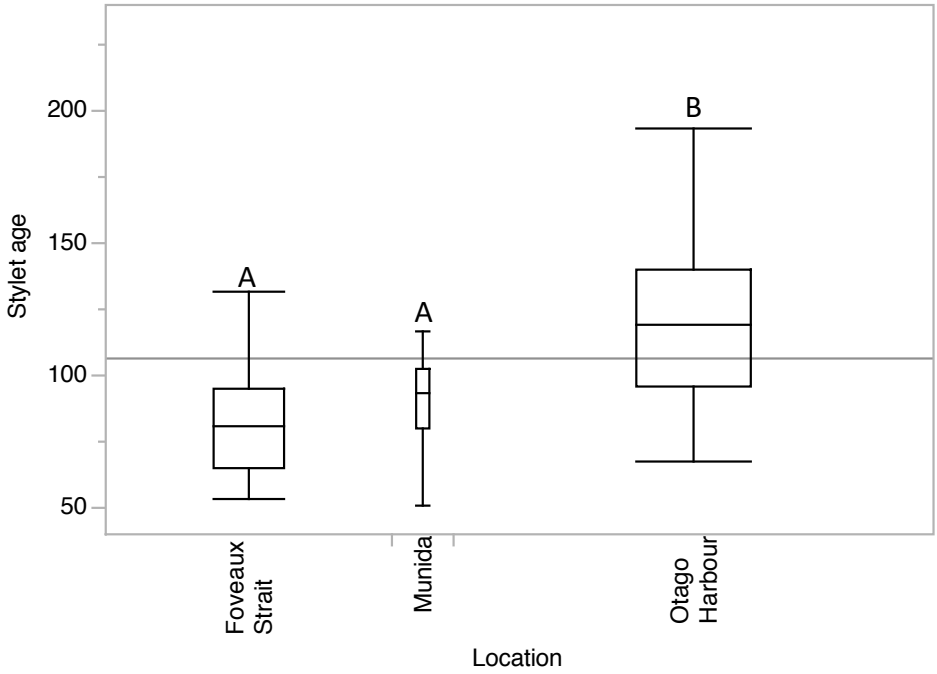

Figure 3.21. Box plot of *Octopus huttoni* stylet age (days) between individuals from Foveaux Strait (n=28), Otago Harbour (n=47), and offshore Otago (Munida)(n=6). The horizontal line in the middle of each box is the median, the upper line is the upper quartile, the lower line is the lower quartile, the upper bar is the maximum value, and the lower bar is the minimum value for that site. The grey horizontal line through the plot represents the median of all of the data. Those indicated by different letters are significantly different ($p<0.05$).

3.3.4 Stylet weight as a proxy for age

Comparisons between stylet weight (mg) and age estimates (stylets and beaks) fit allometric curves (stylet estimate: $y=95.286x^{0.161}$, $r^2=0.34$, beak estimate: $y=-134.64x^{0.210}$, $r^2=0.47$) (Fig. 3.22). When plotted against body weight (g), stylet weight increases allometrically ($y=0.0213x^{1.3128}$, $r^2= 0.77$) (Fig. 3.23). Stylet weight follows the same trend as the other aging methods where individuals from Otago had heavier stylets (0.54-9.48, mean=3.66) than those from Bluff (0.06-2.01, mean=0.64) and individuals from Munida were intermediate (2.42-2.5, mean=2.46) (Fig. 3.24). Stylet weight was significantly different between locations (χ^2 (2, N =52) = 28.51, $p<0.0001$). The Tukey post-hoc further showed that Otago and Bluff were significantly different whereas there was no difference between Munida and the other locations (Fig. 3.24).

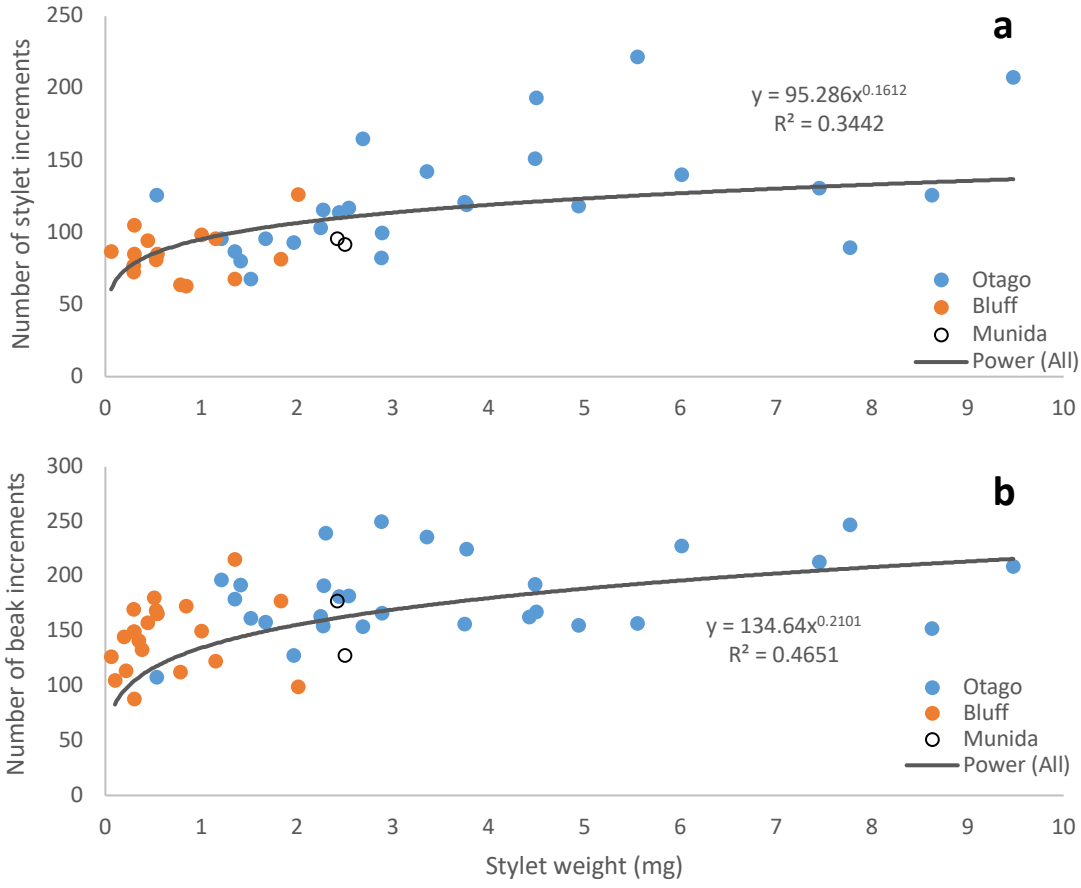

Figure 3.22. Change in stylet weight (mg) as the number of increments in the (a) stylet and (b) beak increases from *Octopus huttoni* from Otago (stylet: n=26, beak: n=29), Bluff (stylet: n=15, beak: n=21), and Munida (stylet: n=2, beak: n=2). Fit with power curves.

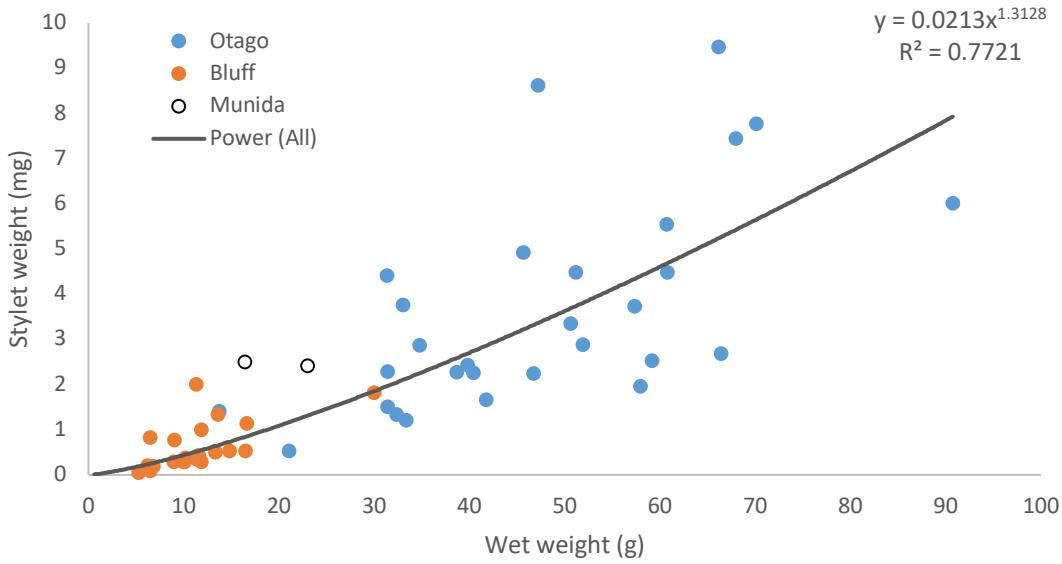

Figure 3.23. Stylet weight (mg) as a function of wet weight (g) for *Octopus huttoni* from Otago (n=26), Bluff (n=15), and Munida (n=2) fit with the best fitting trendline.

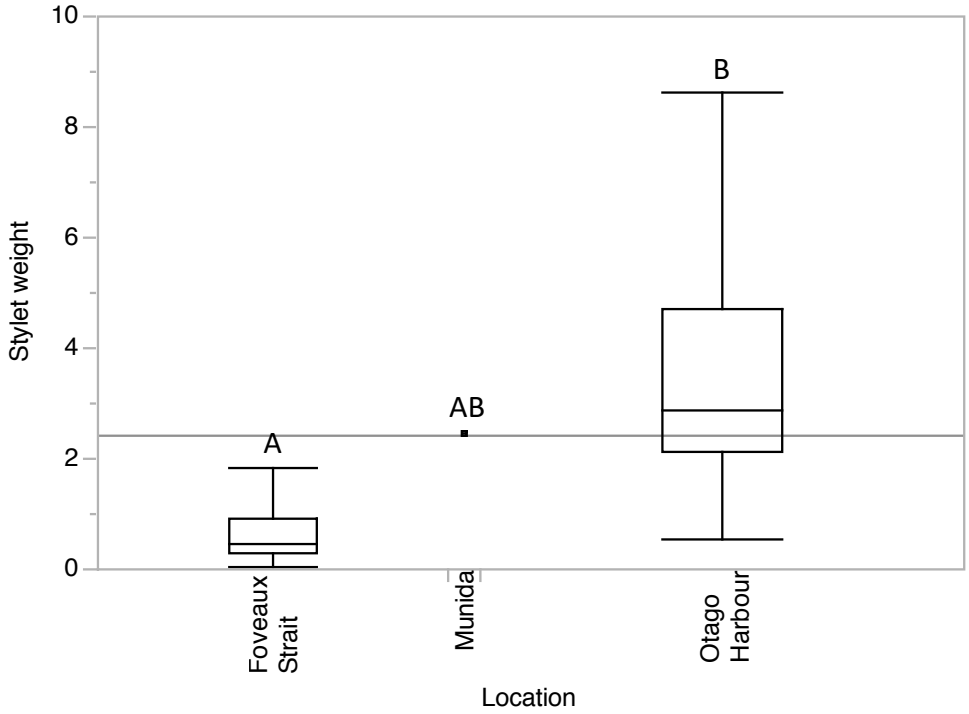

Figure 3.24. Box plot of *Octopus huttoni* stylet weight (mg) between individuals from Foveaux Strait (n=21), Otago Harbour (n=29), and offshore Otago (Munida)(n=2). The horizontal line in the middle of each box is the median, the upper line is the upper quartile, the lower line is the lower quartile, the upper bar is the maximum value, and the lower bar is the minimum value for that site. The grey horizontal line through the plot represents the median of all of the data. Those indicated by different letters are significantly different ($p<0.05$).

3.3.5 Periodicity validation with staining

Stylet staining using a tetracycline bath or by injection produced stylets in which fluorescence occurred throughout the structure without any indication of banding (Fig. 3.25). There appeared to be lines within the fluorescent section, but they were darker. When compared under white light, it was concluded that these "lines" are gap areas where the layers are separating (Fig. 3.25). Leporati and Hart (2015) called these gaps "obstructions". The injection method produced stylets which fluoresced brighter than those stained in the water bath, but there was similarly no indication of fluorescent banding.

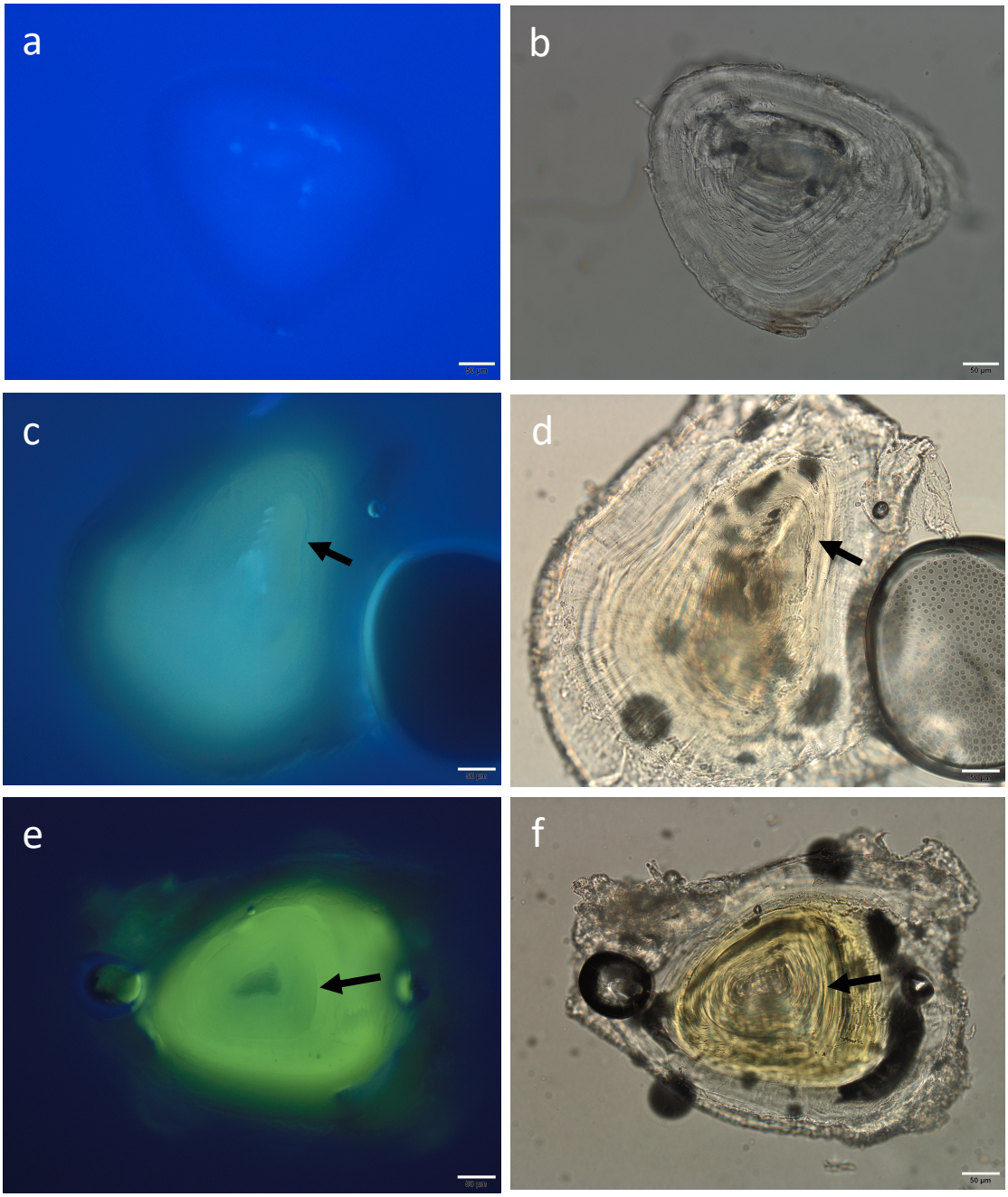

Figure 3.25. *Octopus huttoni* stylet sections showing fluorescence under UV light (a, c, and e) and under white transmitting light (b, d, f). (a) and (b) are micrographs of a section from an individual who had not been stained at all. (c) and (d) are from an individual stained using the tetracycline water bath and (e) and (f) are from an individual stained with injections. All samples were dissected out the same day and prepared the next. (b) was taken with high light exposure and (e) was taken with low light exposure. The black arrows indicate areas where layers are separating. Scale bar = 50 μm.

A linear trend line fit the actual days between staining and death versus the number of counted increments well ($y=0.9931x+0.2988$, $r^2=0.93$) with a 95% confidence interval range from 0.35 to 1.64 (Fig. 3.26). The linear equation with a

slope of 0.99 suggests that growth rings are laid down daily in the stylet. Two spent females were also stained, and increments were counted, but not included in the results as it is believed that the process of laying and brooding eggs affected increment deposition. The actual number of days between the last stain and death was 134 and 147 and the number of counted increments were 45.8 and 41.2 respectively.

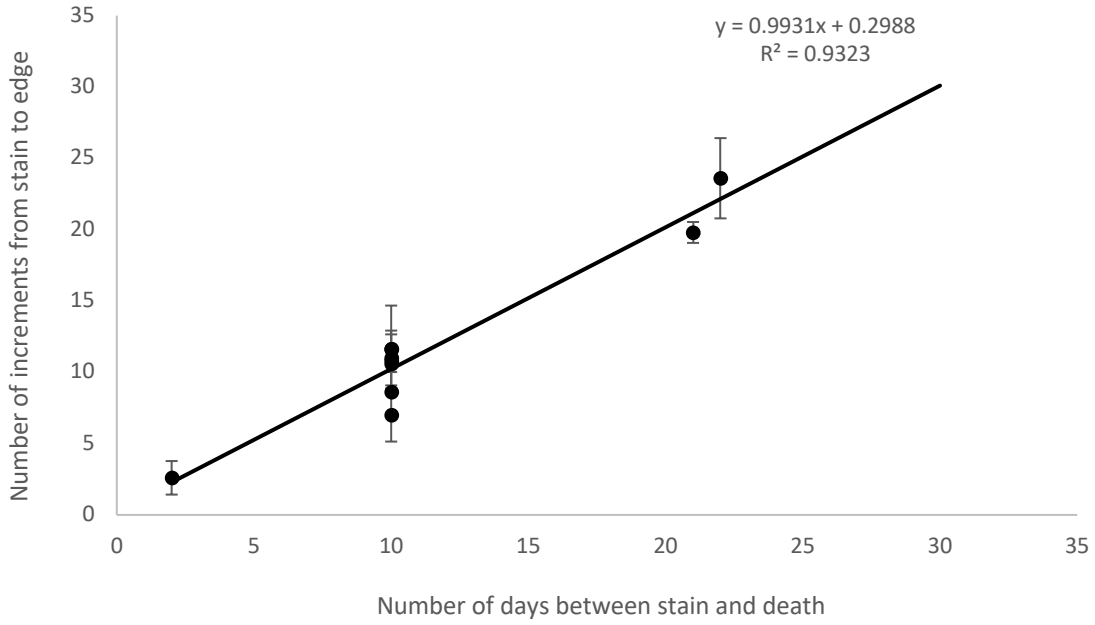

Figure 3.26. Number of *Octopus huttoni* increments between stain and edge of the stylet compared with the actual amount of days passed between staining and death fit with a linear trend line and standard error bars (n=10).

3.3.6 Comparison between stylet and beak increment analysis

When beak and stylet increment estimates were compared, the beak yielded higher ages than the stylet (Fig. 3.27).

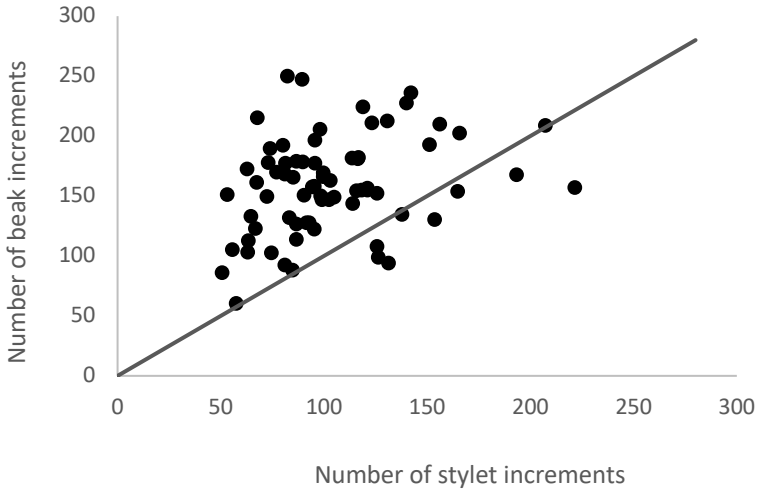

Figure 3.27. Comparison between the number of beak increments and the number of stylet increments of *Octopus huttoni* (n=81) with a 1:1 line to display difference in aging techniques.

3.3.7 Fitting models

Number of beak increments was used as age because stylet increments were difficult to read, therefore total number of increments in each stylet could not be determined with confidence. Both the von Bertalanffy growth model and the Richard's curve fit the age-length relationship well (Richard's r^2=0.688), but the von Bertalanffy curve fit slightly better (r^2=0.690) (Fig. 3.28). The maximum size (L_∞) and growth rate (k) varied between the two models (von Bertalanffy: L_∞=110.40 mm and k=0.0025 mm/day, Richard's: L_∞=58.76 mm and k=0.295 mm/day).

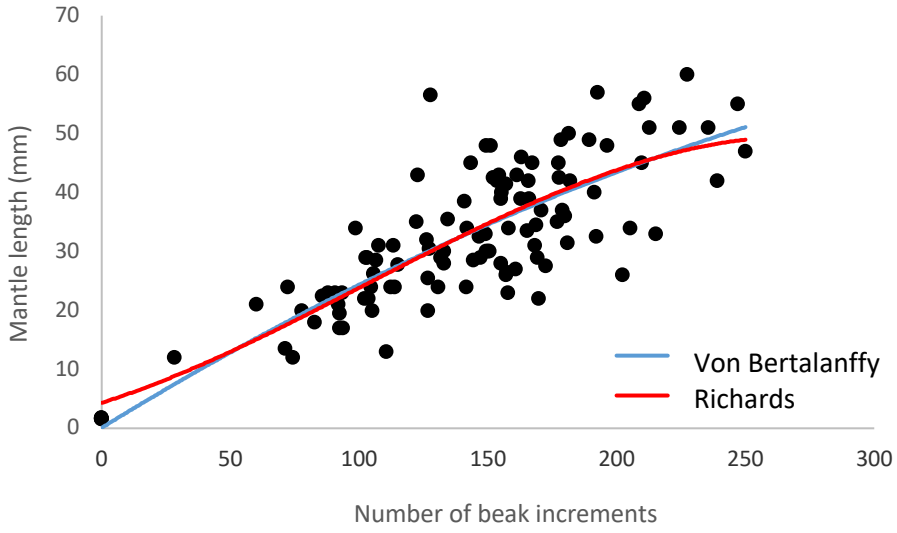

Figure 3.28. Age vs size comparison of *Octopus huttoni* (n=109) fit with a von Bertalanffy growth curve (r^2=0.690) and a Richard's curve (r^2=0.688).

3.4 Discussion

3.4.1 Beak increments

When beaks were cleaned thoroughly with fresh water, increments on the lateral wall were easy to read under 10x magnification on a phase-contrast microscope. Increments on the rostrum under the hood were harder to read because of the thickness, but an increase in light often solved this problem. Hernandez-Lopez *et al*. (2001) also had difficulty counting increments on the rostral tip because of the high pigmentation caused by the thickness of this area. Some erosion was observed on *O. huttoni* rostral tips most likely from predation on crustaceans, which could cause some underestimation but not enough to greatly affect the count. Even with erosion, Perales-Raya *et al.* (2014) found that the lateral wall surfaces method is a more accurate indicator of chronological age than other methods.

Comparisons between the size of the individual (weight and mantle length) and the beak age estimate was best represented by positive allometry. Perales-Raya *et al*. (2010) also found that an allometric curve best fits weight versus beak age estimated from the lateral wall of *O. vulgaris*. However, Cucu *et al.* (2013) found an exponential curve to best fit *O. vulgaris* beak estimates. In all instances, an upwards curve was found indicating that the present data is comparable to published studies.

The number of beak increments were significantly different between individuals from Otago and Bluff, but not Munida. Therefore, Otago and Bluff represent two different age populations with those caught in Bluff being younger than those caught in the Otago Harbour. The hypothesis that this species experiences an ontogenetic shift in habitat in which offshore populations are comprised of younger individuals and as they mature, they make their way inshore to spawn is supported by these results. To reiterate, this hypothesis came about because Stranks (1996) suggested that small egg species such as *O. huttoni* could be transported offshore as planktonic young by inshore coastal currents. In addition, Otero *et al*. (2009) suggest that areas of upwelling also have the potential to transport larvae to the open ocean. González *et al*. (2011) and Mayo-Hernádez *et*

al. (2013) then suggest that *O. vulgaris* adults migrate inshore to spawn in the shallows and that immature specimens predominate the depths. Because those from Munida were not statistically different from Otago or Bluff, we can hypothesise that they represent the younger portion of the Otago population, but are not as young as those caught in Bluff. To further validate this hypothesis, more samples from Munida and an additional site from inshore Bluff should be analysed so that offshore and inshore individuals from Bluff can be compared in size and age.

When size (mantle length) at age (beak age estimates) data were compared between the present study and the 2004-2006 dataset, the 2004-2006 beak increment estimates were not significantly different from Bluff individuals in the present study. This was expected because all except three individuals from the 2004-2006 dataset were from Bluff and the methodology was the same. This also gives further evidence that the Bluff population is comprised of younger individuals than those in the Otago Harbour.

3.4.2 Beak length as a function of age

Beak length increased with mantle length and beak age estimate exponentially. When compared among locations, beak length was found to be significantly different between Otago, Bluff, and Munida. As with the beak age estimates, this indicates that these three locations could represent separate ontogenetic populations because beak length is closely correlated with age. These relationships between beak length and size and age of the individual is useful in prey studies. *O. huttoni* is prey for many kinds of marine animals such as fish, penguins, and marine mammals and because beaks normally survive the digestion process, they can be used to estimate prey size (Lalas, 2009; Lalas & Webster, 2013).

3.4.3 Increment validation using paralarvae beaks

Paralarval beak halves were identified as upper or lower by using the description of *O. vulgaris* paralarvae beaks by Franco-Santos *et al.* (2014). The absence of a slit between the two halves of the jaw identify the upper beak and the presence of

teeth across the entire side of the beak identify the lower beak (Franco-Santos *et al.*, 2014). Franco-Santos *et al.* (2016) was used as a guide to look for increments in the area in which, they propose increments are laid. The paralarval beaks from this study appear to be less developed (have more teeth across the whole rostrum and more transparent) than the *O. vulgaris* beak that Franco-Santos *et al.* (2016) present, although they were around the same age (12 and 15 days old). This could be because *O. vulgaris* (Franco-Santos *et al.*, 2016) have slightly larger paralarvae at hatching (1.95 mm) compared with *O. huttoni* (1.72 mm) and therefore more developed beaks.

There were clusters of short lines fanning out from one point in a few 12-day old beaks, but the count was more than 20 suggesting that these were not increments. Besides the line clusters, no growth rings were found suggesting that this species does not lay down rings during this time in their life. The smallest number of increments that were counted on a beak during this study was 28 from a juvenile male from Bluff (ML= 12 mm, WW=0.603 g). This juvenile was small, but if Carrasco (2014) is correct in that *O. huttoni* probably spend from 40-70 days as planktonic paralarvae, this small juvenile would be older than 28 days old. Because no increments were found on paralarval beaks, as old as 12 days old, and the youngest juvenile only had 28 growth increments, it can be hypothesised that the beak does not lay down increments during the paralarval stage. Beak increments may start forming after settlement when the shift from pelagic to benthic occurs and they start to eat larger prey. To solidify this hypothesis, known-age individuals between the sizes of young paralarvae (12 days) and recently settled juveniles need to be sampled. But from the data presented, there is a possibility that increments are not laid down during the planktonic stage indicating that beak estimates are only representative of their age after settlement.

If the first increment on the beak does not form until settlement, the back calculation using number of beak increments represent a majority of *O. huttoni* individuals settling in spring. If increments start forming immediately post hatching, then these results represent the number of individuals that have hatched during this time. Other species of octopus such as *M. maorum* (Anderson, 1999),

Octopus cyanea (Herwig *et al.*, 2012), and *Octopus magnificus* (Smith *et al.*, 2006) have been shown to also have a peak in spawning and recruitment during spring/summer when temperatures are increasing. Pecl (2004) found that at a given length, southern calamary, *Sepioteuthis australis*, individuals that hatched in spring/summer had heavier mantles than those hatched in winter/autumn. This is most likely because the rise in temperatures and increase in prey availability increases juveniles growth rates and survivability (Pecl, 2004).

3.4.4 Stylet increments

Like stylets of the similar merobenthic octopus *M. maorum* (Doubleday *et al.*, 2011), the most difficult area to read was the nucleus or pre-hatch region.

Stylet age estimates compared with mantle length and weight of the individual displayed positive allometric trends. These trends show a relationship between estimated age and the size of the animal, but there was too much variation to be certain. When stylet increment estimates were compared between locations, there was a significant difference, specifically between Otago individuals and the other two locations. Munida and Bluff individuals were not significantly different in stylet increment estimate, which is similar to the results for beak increment estimates as individuals from Munida were not significantly different from either location. This further verifies that the Munida population is comprised of individuals of intermediate ages.

3.4.5 Stylet weight as a function of age

Stylet weight compared to age estimated from stylets and beaks was best described by positive power functions. When weight of the individual was compared with stylet weight, a power trend was also observed. This is comparable to the trend that Leporati and Hart (2015) observed with a similar merobenthic octopus, *O. tetricus*. Even the slopes were similar in that Leporati and Hart (2015) reported slopes of 1.265 for females and 1.435 for males and the slope in the present study was 1.313.

3.4.6 Increment validation using tetracycline staining

Staining more than once proved to be unnecessary since the entire stylet would stain and there were no noticeable fluorescent lines like those seen in statoliths. This may be due to the stylet being more absorbent and softer than a squid staolith in which actual fluorescent lines were produced from the stain. However, once the stylet was stained, the increments from the last stained edge to the edge of the stylet could be counted and used for periodicity validation. Leporati and Hart (2015) also used this method to validate daily deposition in *O. tetricus* stylets using calcine injected animals. The trend line showed a ring periodicity of one ring per day (i.e. a slope of 0.99), validating daily growth rings.

There were two females which were stained twice using the water bath method and then laid and brooded eggs. There were 134 and 137 days between the second stain and death for these two females and 45.8 and 41.2 increments were counted respectively. These females were not feeding for 169 days (as part of their natural brooding behaviour), which could explain why the increment count was so low. It should be noted that daily increment deposition was validated with regularly fed octopus. Therefore, deposition is daily as long as food is abundant. Food intake affects the growth of animals and could therefore affect the disposition rate of growth rings in the stylet. Leporati and Hart (2015) also had females that laid eggs and could not count lines between the stain and death and therefore determined that they may stop laying down increments just before laying. If this is true, the counts from the two females from this study would represent the number of days from the first stain to the egg laying date (28 and 25 days) which is more accurate than the number of days between the second stain and death.

3.4.7 Applicability of growth models

Beak age estimates were used for modelling size at age growth as they were determined to be closer to actual age than stylet age estimates. The von Bertalanffy growth model was the best fit to the age-length data according to the r^2 value, but it could be argued that both models fit just as well (r^2 are similar). The Richard's curve is more realistic as the von Bertalanffy curve predicts a maximum mantle

length of 110 mm, which is not realistic for this small species of octopus. Although these models can be fit to the data, they might not be the best for modelling cephalopod growth because they both assume the presence of asymptotes where a maximum size would be reached at the end of their life span. Cephalopods are known for having non-asymptotic growth, therefore, these models might not be appropriate. A model that does not assume presence of asymptotes and takes the two-phase growth pattern into account would be better suited to model growth in cephalopods.

3.4.8 Aging methodology

This study aimed to test various methods for estimating the age of *O. huttoni* in southern New Zealand. Beak and stylet increments both gave age estimates, but only stylet periodicity could be validated as daily. Although stylet increments were daily, the nucleus was notoriously hard to read, and the first increment could not be confidently determined. This suggests that the stylet may be an underestimate such as the beaks (Doubleday & Semmens, 2011). When both increment counting methods were plotted against one another, beaks produced higher estimates than stylets. This shows that although there may be an underestimation in beak count because of erosion and the possibility that increments are not deposited until settling, the difficultly in reading the stylet may produce a more severe underestimate. If one of these two methods had to be chosen over the other, I believe that beak increment analysis provides a more accurate estimate of age than stylet increment analysis in *O. huttoni.* This is due to the difficulty in readability as in almost all stylets because there were large portions which could not be counted. Doubleday *et al.* (2011) also had variable stylet age estimates in the merobenthic octopus *M. maorum*. They determined that this species could not be aged using stylet increment analysis due to the fact that these estimates suggest non-feasible growth rates (Doubleday *et al.*, 2011). *M. maorum* stylets are also soft like the *O. huttoni* stylet suggesting that the texture makes it difficult to prepare and count. Doubleday *et al.* (2011) hypothesize that in merobenthic species, such as *M. maorum* and *O. huttoni,* the stylet forms post-hatch and maybe even as late as settlement. If this is the case, stylet estimates would be largely underestimated.

Although I have validated daily increment disposition in the stylets of adults, nothing is known on the age of first increment formation of this merobenthic species. Therefore, these estimates cannot be considered absolute age. The pros and cons of each aging method is summarised in Table 3.3 below.

Table 3.3. Summary of the pros and cons of each aging method; beak increment analysis, stylet increment analysis, stylet weight, and beak length.

Method	Pros	Cons
Beak increment analysis	• Easy to prepare and increments easily visible • Correlates highly with size of individuals	• Small beaks are brittle and easy to crack when mounting with warm Crystalbond™ • Need known-aged individuals to validate periodicity
Stylet increment analysis	• Possible to validate periodicity with tetracycline staining in wild-caught animals	• Difficult and long to prepare • Soft consistency of stylet makes it easy to create obstructions • Nucleus is notoriously difficult to read
Stylet weight	• Fast and easy to measure	• Lots of variability • Does not estimate age
Beak length	• Fast and easy to measure • Correlates highly with size of individuals	• Does not estimate age

3.4.9 Conclusions

In conclusion, beak increment analysis was found to be the best aging method as estimates were highly correlated with the size of the individuals. Stylet incrementation was validated as daily in the adults measured, but because the nucleus is difficult to read, these age estimates were determined to be underestimates. Both size at age models fit the data just as well, but the von Bertalanffy suggested an unrealistic maximum size. Therefore, the Richard's curve was determined to be a better fit. More data on juvenile samples from paralarvae to maturity is needed to visualize if this species displays two growth phases and if increments on beaks are deposited post settlement. If this is true, beaks may only be representative of the age post settlement. These age estimates further support the findings that Otago individuals are older than Munida and Bluff individuals further supporting the ontogenetic shift hypothesis. Overall, this study provided valuable information on the different aging methods and their applicability for wild-caught *O. huttoni,* offering a baseline for future research.

Chapter 4: Lipofuscin

4.1 Introduction

4.1.1 How is lipofuscin formed?

Age pigments or lipofuscin granules are a metabolic bi-product of respiration that bind to postmitotic cells, and cannot be degraded nor ejected (Gray & Woulfe, 2005; Terman & Brunk, 2004; Sheehy, 2002b). Aerobic metabolism continuously produces oxygen radicals and hydrogen peroxide as bi-products which can then cause oxidative damage to proteins, lipids, and nucleic acids (Zielinski & Portner, 2000). This occurs when peroxisomes and mitochondria produce superoxide ($O_2^{\bullet -}$) which is then converted to hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase (SOD) (Equation 1 & Fig. 4.1) (Zielinski & Portner, 2000). Most H_2O_2 is enzymatically broken down to water by catalase and glutathione peroxidase, but some may enter the autophagolysosome (Terman & Brunk, 2004) (Equation 2 & 3, Fig. 4.1).

(Equation 1) $O_2^{\bullet -} + 2H_2O \rightarrow 2H_2O_2$

An extremely reactive hydroxyl radical ($HO^{\bullet}$) derived from oxygen is then formed from the reaction between H_2O_2 and ferrous iron (Equation 2)(Terman & Brunk, 2004). These highly reactive hydroxyl radicals attack anything they meet and do not diffuse because they have a half-life on the order of 10^{-9} making these reactions site specific (Terman & Brunk, 2004). These principles are demonstrated in Figure 4.1.

(Equation 2) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\bullet}$

(Equation 3) $LH + HO^{\bullet} \rightarrow L^{\bullet} + H_2O$

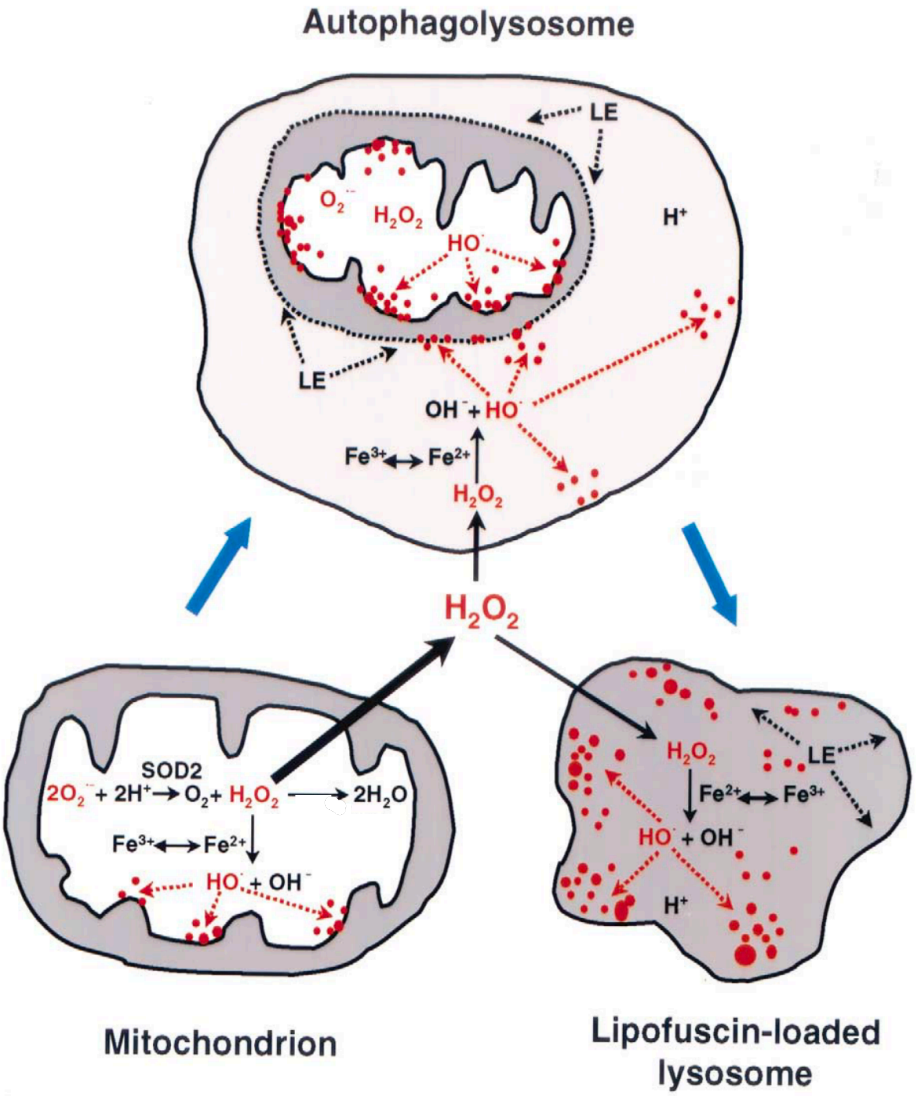

Figure 4.1. Schematic showing the reactions leading to the formation of lipofuscin. The blue, bold arrows indicate the sequence of events. The red dots are oxidatively damaged macromolecules, the black dotted arrows symbolise action of lysosomal enzymes (LE) and the red dotted arrows symbolise action of the HO• radical. (Adapted from Brunk and Terman, 2002)

If HO• reacts with unsaturated lipids (L), a chain reaction occurs which results in aldehydes, specifically malondialdehyde (MDA), which can be used as an indicator of age and oxidative stress in cephalopods (Equations 3-6) (Zielinski & Portner, 2000; Terman & Brunk, 2004).

(Equation 4) $L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$

(Equation 5) $LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$

(Equation 6) $LOOH \rightarrow$ various aldehydes such as MDA

Schiff bases are then formed from the reaction between these aldehydes and protein residues containing one or two amino groups. These Schiff bases undergo intra-molecular rearrangements (Terman & Brunk, 2004). Plastic-like compounds are then formed when various protein residues are cross-linked by aldehyde bridges, resulting in lipofuscin aging pigments (Terman & Brunk, 2004). Lipofuscin is resistant to lysosomal recycling and therefore accumulates over time with age, which is why, more recently, it has been used for aging on animals that do not have reliable aging methods such as crustaceans (Maxwell *et al.*, 2007; Sheehy, 1990a, 1990b, 1989).

4.1.2 Lipofuscin as an aging method

Lipofuscin has been a successful indirect method of estimating age of marine animals such as crustaceans(Sheehy, 1990a; Maxwell *et al.*, 2007; Sheehy, 1992) and the octopus *Octopus pallidus* (Doubleday & Semmens, 2011). When used as an aging method, lipofuscin can be detected in postmitotic cells, such as neurons, using three histochemical methods; observing unstained histological sections of formaldehyde fixed tissue under a fluorescent microscope, counting pigment granules in stained (ex: Sudan black) histological sections, and using a spectrofluorometer to measure the fluorescent intensity of dissolved lipofuscin (Hammer & Braum, 1988). The first two methods are the most common, so for this study unstained histological sections were viewed under a fluorescent microscope.

A variety of stains can be used to identify lipofuscin granules such as Sudan black and periodic acid-Schiff (PAS). Sudan black stains lipids black and PAS stains glycogen and other carbohydrates magenta, nuclei blue, and cytoplasm light pink (D'Angelo *et al.*, 1956; Evangelou & Gorgoulis, 2017). The PAS method identifies lipofuscin by being PAS positive, bright magenta spots which are acid-fast (D'Angelo *et al.*, 1956). When stained granules are compared to those unstained under fluorescence, they can be verified as lipofuscin pigments.

Any tissue that contains postmitotic cells can be used for lipofuscin analysis, but areas with high amounts of neurons may be more suitable such as the brains in

crustaceans (Sheehy, 1989, 1990b) or the optic lobes in octopus (Doubleday & Semmens, 2011). In postmitotic cells of short-lived species, lipofuscin accumulates more rapidly than long-lived species (Terman & Brunk, 2004). This is because metabolism causes oxidative stress, which is the cause of lipofuscin loading in cells. Therefore, a high metabolic rate may have a negative impact on an animal's life span (Terman & Brunk, 2004; Zielinski & Portner, 2000). This has been proven to be true in mammals as well as in cephalopods *Sepia officialis* and *Lolliguncula brevis* as cephalopods typically have high metabolisms and short life spans (Zielinski & Portner, 2000). A few studies have found that lipofuscin accumulation rate is correlated with metabolic rate (Zielinski & Portner, 2000; Sohal & Donato, 1979; Sheldahl & Tappel, 1974) therefore, we would expect that the high metabolic rates in octopus would cause a large accumulation of lipofuscin.

As with any aging technique, environmental factors can affect the accuracy of the age estimate. In lipofuscin aging, temperature could affect the accumulation rate because it affects metabolism (Arkhipkin *et al.*, 2018). For this reason, a number of researchers suggest that it may not be an accurate estimation of direct age in cephalopods and is probably more useful to identify relative age as in age cohorts (Arkhipkin *et al.*, 2018; Zielinski & Portner, 2000; Doubleday & Semmens, 2011).

4.1.3 Aims

The aim for this study was to determine the applicability of lipofuscin as an aging method in wild-caught midget octopus, *O. huttoni.* To accomplish this, lipofuscin volumes were compared to size and estimated age. Lipofuscin volume ratios were estimated for individuals from Bluff and Otago and values were compared between populations.

4.2 Methods

Methods on capture and euthanasia of octopus are located in Chapter 2. Because lipofuscin as an aging method is relatively new, it is important to standardize the samples by using known-aged animals first to get an idea of the accumulation rate.

However, as known-aged animals were not available for this study, wild caught octopus were used, and estimated age was used instead.

4.2.1 Histology

Optic lobes from 59 octopus from Bluff and 47 from Otago were dissected out and analysed for lipofuscin using histological methods. Immediately after dissection, the optic lobes were fixed in FAACC (formalin-acetic acid-calcium chloride) for 48 h and then preserved in 70% ethanol at least 24 h before analysis. FAACC was made with 400 mL 10% formalin, 13 g of calcium chloride dihydrate, 50 mL of glacial acetic acid, and 550 mL of distilled water. Optic lobes were then taken to the histology lab at the University of Otago Zoology Department where they were dehydrated in 95% ethanol for one hour and then twice in 100% ethanol for one hour each. The tissues were cleared twice in xylene for 40 minutes each. Next, they were imbedded in paraffin wax by soaking in warm wax in a 60°C oven for three rounds of 40 minutes each, changing the wax in between each 40 minutes. They were then imbedded in blocks of paraffin and left overnight to set. After setting, the blocks were placed on a cold tray in the freezer for ~3 minutes and removed from the mould. Blocks were stored in the fridge and when taken out, kept face down on a cold tray to prevent the wax from softening. A microtome (Leica RM2125RT) was used to cut 3-4 thin sections (5 μm) per sample. The sections were placed on the microscope slide using cold (16°C) and warm (45°C) water baths and left on a warm plate (45°C) overnight. Xylene baths were used to dissolve the wax from the slides and coverslips were mounted using Entellan® mounting medium. Because lipofuscin is highly auto fluorescent, no stain was necessary. A second slide per sample was prepared and stained with Hematoxylin and Eosin (H&E) for a visual reference of the tissue.

Additional procedures were done using Sudan black as a stain and a modified periodic acid-Schiff (PAS) to validate that fluorescent granules were actually lipofuscin. This was done for two samples with the highest beak age estimate, two samples with the lowest, and one median. These were done by the laboratory technicians at the histology lab in the Pathology Department, University of Otago.

4.2.2 Quantifying volume ratio

An epifluorescence microscope (Olympus BX51) with a camera (Olympus XC50) and excitation filter of 450-490nm (emission of 510nm) attached to a mercury lamp (Olympus U-RFL-T) was used to view and take micrographs of 10 random fields of view per sample. The volume ratio of lipofuscin granules was then measured using the digital micrographs in the image analysis program, Image J. Average volume ratio (%) and coefficient of variation (CV) was calculated for each sample.

In JMP Pro (version 11.0), Shapiro-Wilks tests were applied to test for normal distribution for the two sites (Otago and Bluff) and a chi-squared test was used to test for significant differences between lipofuscin volume ratio between sites. A regression model was fit in JMP to test the relationship between lipofuscin and age (beak estimates), weight, mantle length, and total length.

4.3 Results

4.3.1 Identification of lipofuscin granules

The H&E stained tissue showed an aggregation of blue/purple spots on the outskirts of the optic lobe with an "empty" band (pale pink) separating the outer and inner portion of the lobe (Fig. 4.2). These blue/purple spots represent the nuclei of cells and the pale pink represents connective tissue (Fig. 4.2).

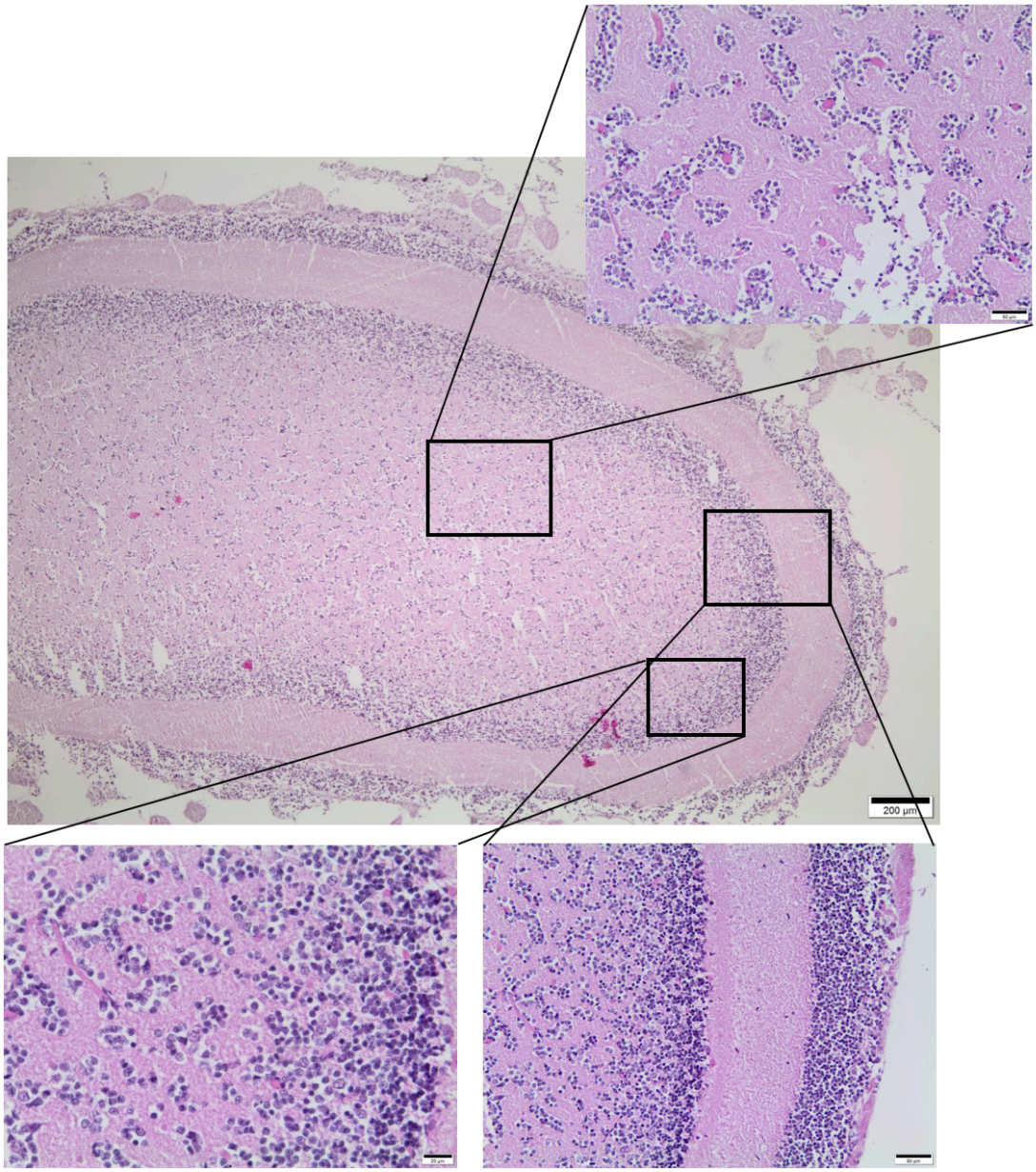

Figure 4.2. Hematoxylin and eosin stained *Octopus huttoni* optic lobe with zoomed in portions showing the uneven distribution of neurons and the "empty" band that surrounds the lobe. Scale bar = (a) 200 µm, (b and d) 50 µm, (c) 20 µm.

The Sudan black and PAS stain were successful in identifying lipofuscin granules (Figs. 4.3).

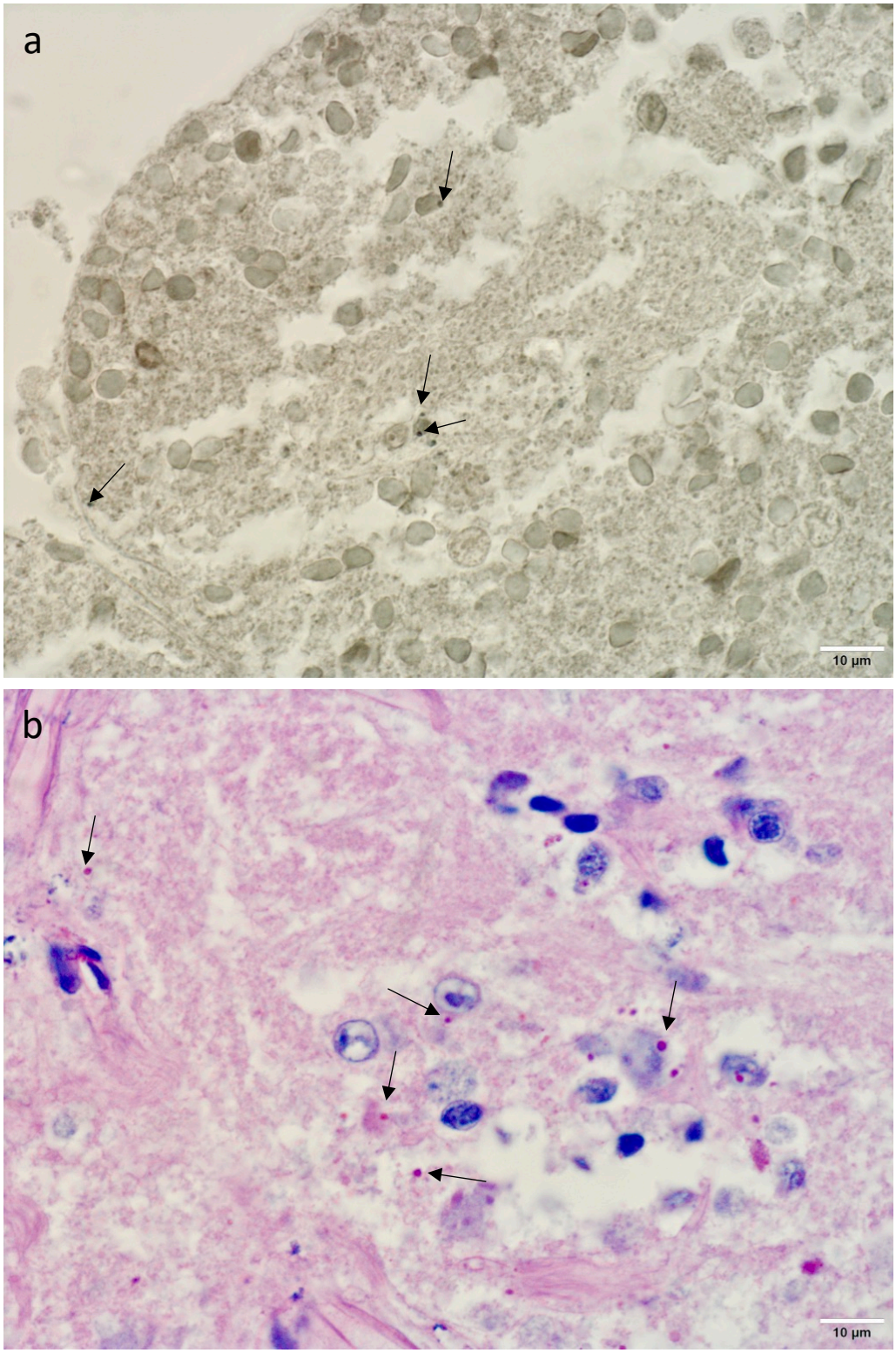

Figure 4.3. Histological section of *Octopus huttoni* optic lobe (estimated age: 236 d) stained with Sudan black (a) and periodic acid-Schiff (PAS) stain (b). In the Sudan black stain, lipofuscin granules are stained black, nuclei are stained dark grey, and connective tissue is stained light grey. In the PAS stain, lipofuscin granules are stained bright pink (magenta), nuclei are stained blue-purple, and connective tissue is stained light pink. Some lipofuscin granules are indicated by black arrows. Scale bars = 10 µm.

The location and size of fluorescence granules are also consistent with lipofuscin characteristics as they are associated with cell membranes and are ~1-5 μm (Fig. 4.4).

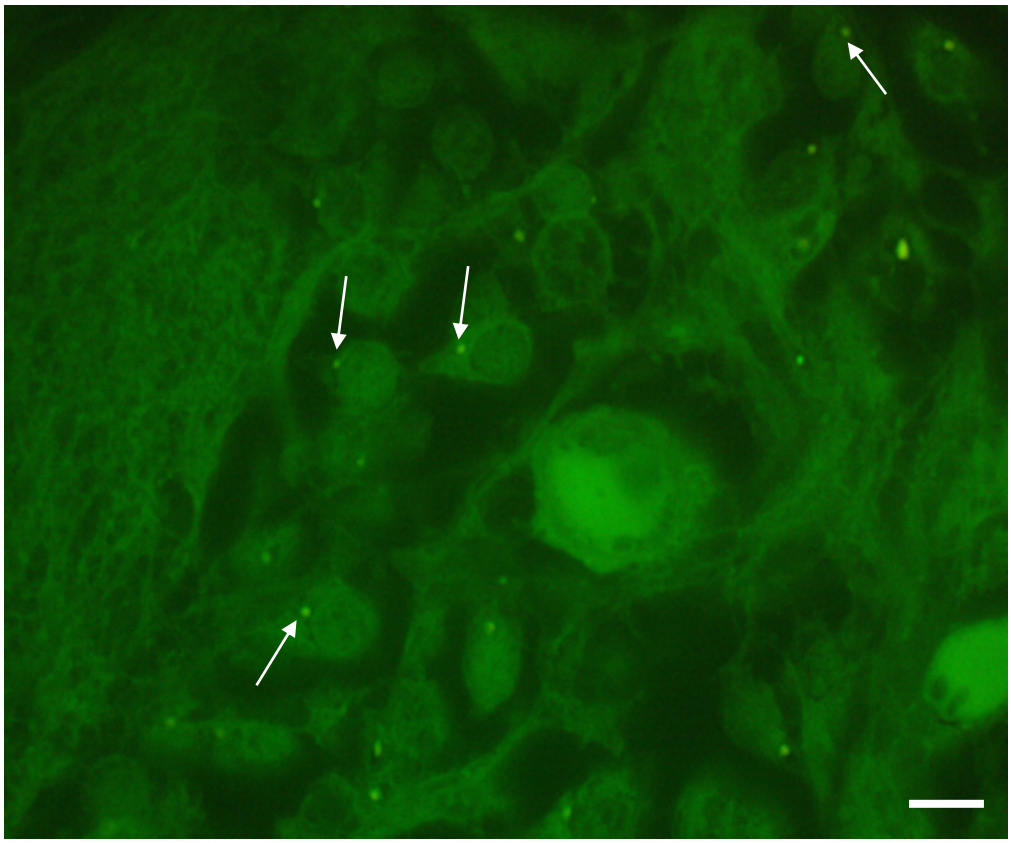

Figure 4.4. Unstained histological section of *Octopus huttoni* (estimated age: 155 d) optic lobe showing the fluorescence (450-490 nm emission filter) and location of lipofuscin granules. Examples of lipofuscin are indicated by the white arrows. Scale bar = 10 μm.

4.3.2 Lipofuscin volume between locations

The Shapiro-Wilks tests showed that lipofuscin volume ratio was not normally distributed for Otago individuals ($p=0.0001$) nor Bluff ($p<0.0001$). The chi-squared showed that there was a significant difference between the two populations in terms of lipofuscin volume ratio in which Otago individuals had slightly larger volumes than those from Bluff (χ^2 (1, N=85) =5.2814, $p=0.0216$) (Fig. 4.5 & 4.6).

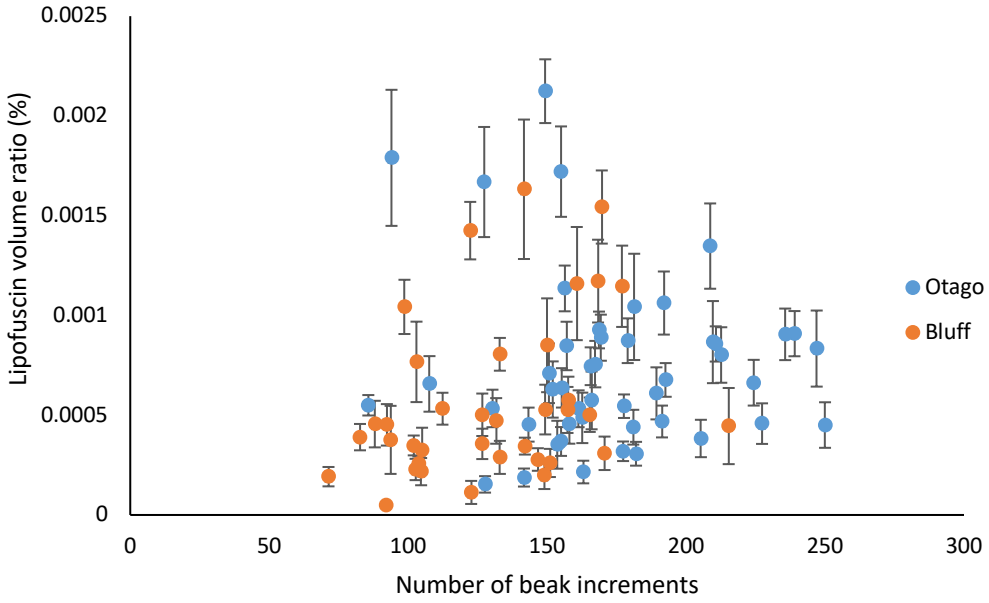

Figure 4.5. Comparison of the number of beak increments to lipofuscin volume ratio (%) of *Octopus huttoni* from Otago (n=47) and Bluff (n=59) with standard error bars.

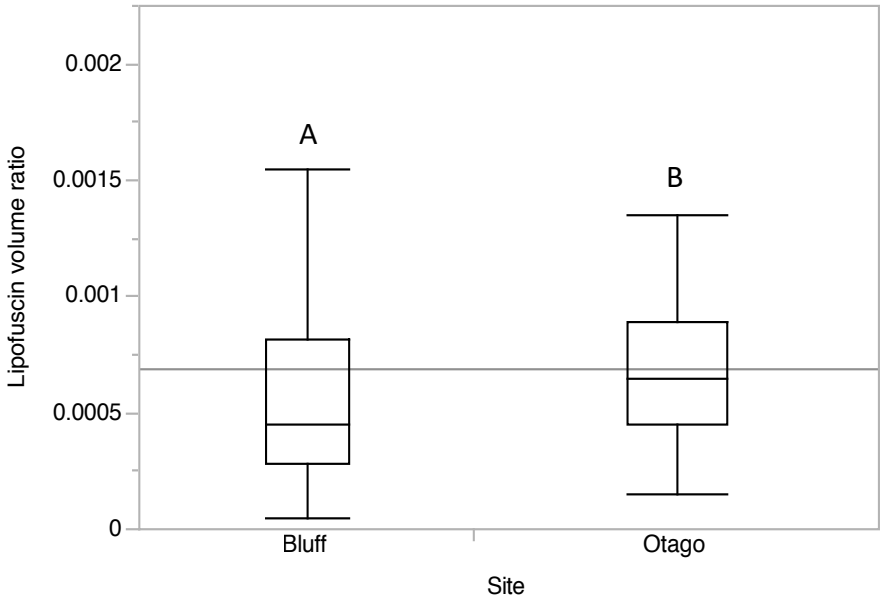

Figure 4.6. Box plot of lipofuscin volume ratio in *Octopus huttoni* optic lobes between sites (Otago (n=47) and Bluff (n=59)). The horizontal line in the middle of each box is the median, the upper line is the upper quartile, the lower line is the lower quartile, the upper bar is the maximum value, and the lower bar is the minimum value for that site. The grey horizontal line through the plot represents the median of all of the data. Those indicated by different letters are significantly different ($p<0.05$).

4.3.3 Lipofuscin as a function of animal size and estimated age

Lipofuscin volume ratio appeared to increase with increasing body weight ($y=0.0003x^{0.1755}$, $r^2=0.049$), total length ($y=4E\text{-}05x^{0.5133}$, $r^2=0.060$) and mantle length ($y=0.0002x^{0.3592}$, $r^2=0.029$) (Fig. 4.7). There also appeared to be an increase in lipofuscin volume with age estimates from stylets (power: $y=1E\text{-}05x^{0.76}$, $r^2=0.096$; exponential: $y=0.0003e^{0.0049x}$, $r^2=0.086$) and beaks (power: $y=1E\text{-}05x^{0.8488}$, $r^2=0.154$' exponential: $y=0.0003e^{0.0078}$, $r^2=0.141$) (Fig. 4.8). However, the regression analysis showed no significant relationships between mantle length ($p=0.972$), total length ($p=0.167$), weight ($p=0.302$), or age (beak estimate $p=0.632$, stylet estimate $p=0.218$) and lipofuscin.

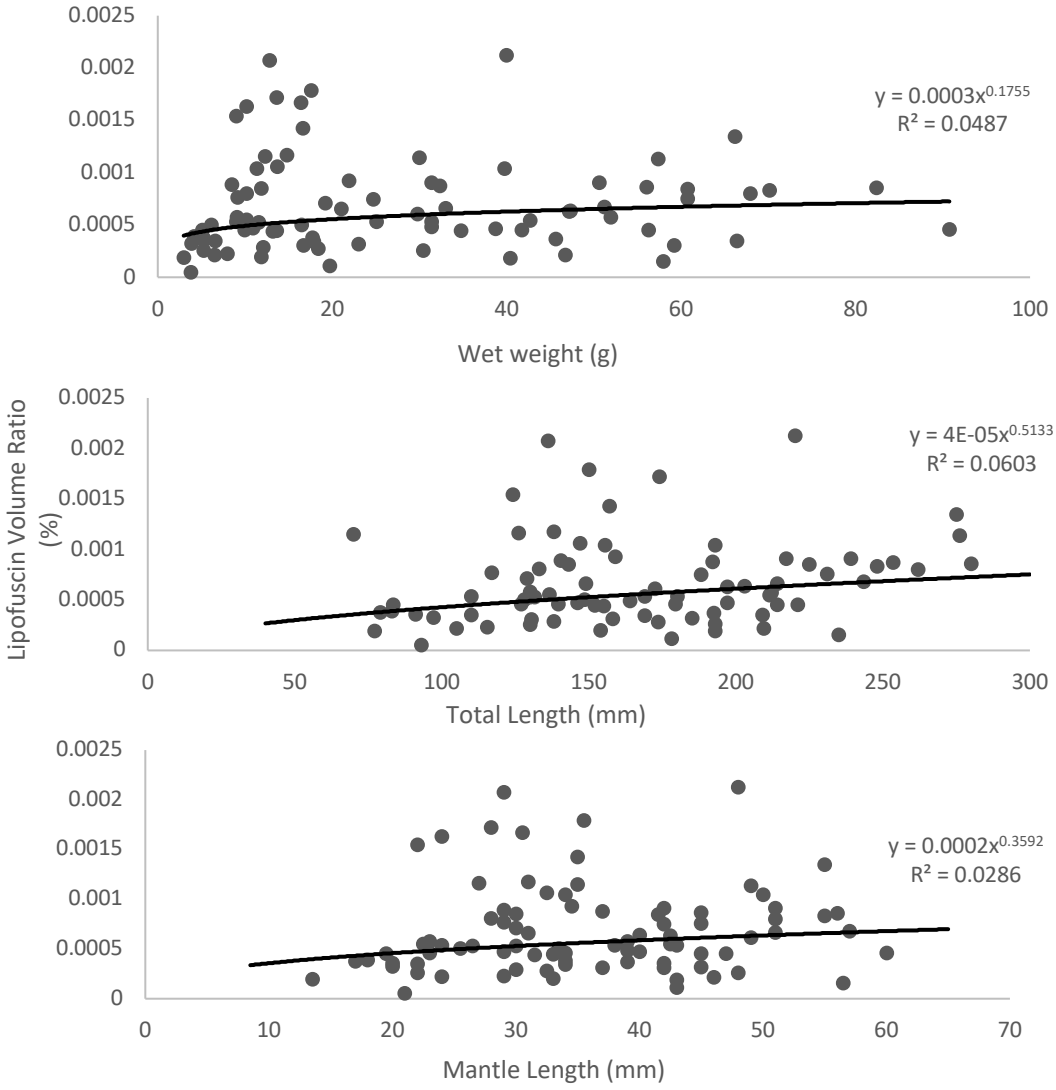

Figure 4.7. Comparison between lipofuscin volume ratio (%) and wet weight (g), total length (mm), or mantle length (mm) of 106 *Octopus huttoni* fit with power curves.

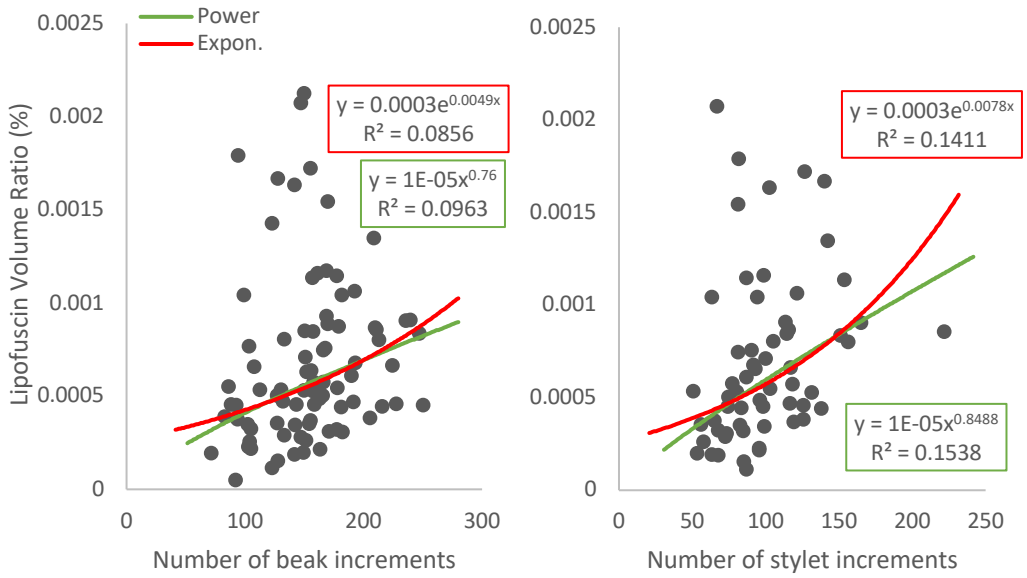

Figure 4.8. Comparison of the number of beak increments and number of stylet increments from 106 *Octopus huttoni* to lipofuscin volume ratio (%) fit with power curves (green) and exponential curves (red).

4.4 Discussion

4.4.1 Distribution and appearance of lipofuscin granules

There was an aggregation of nuclei on the outer sides of the optic lobes and an absence of nuclei which formed a band around the outer portion of the lobes. There were noticeably fewer nuclei and more connective tissue in the centre of the lobe when compared to the outer region indicating that cells are unevenly distributed. If these nuclei represent cells that contain lipofuscin granules, then the granules are also unevenly distributed. Maxwell *et al.* (2007) reported granules aggregating on the outer margin in Caribbean spiny lobster eyestalks, but the cause of this uneven distribution is unknown. The Sudan black and PAS stain identified that lipofuscin granules are associated with these nuclei as they are bound to the membrane of cells (Brunk *et al.*, 1992; Gray & Woulfe, 2005; Bluhm *et al.*, 2001).

No pattern in lipofuscin distribution was observed from the stained sections, but because nuclei are unevenly distributed and lipofuscin were not found in the

connective tissue, it is hypothesised that lipofuscin are as well. If there is an uneven distribution of lipofuscin granules, more micrographs per sample would need to be analysed. This study sampled 10 random field of view per individual, but some studies suggest using 20-30 to get a more precise reading (Maxwell *et al.*, 2007; Sheehy, 2002a).

Granules were verified as lipofuscin by using the Sudan black and PAS stain, which stained lipofuscin dark. The location and size of the granules under fluorescence are also consistent with lipofuscin characteristics. Confirming that granules which were analysed were, in fact lipofuscin.

4.4.2 Lipofuscin volume between Otago and Bluff

Lipofuscin volume ratio was significantly different in individuals from Otago and Bluff, where volume in Otago samples was greater. This is consistent with the results from the previous chapter where it was found that Otago individuals were significantly older than those from Bluff. These results provide support for the hypothesis that lipofuscin can be used as an aging method in octopus optic lobes.

4.4.3 Accumulation of lipofuscin granules as a function of size

There was an increase of lipofuscin with weight and length expressed by power curves, but the statistics showed that there was no significant relationship between lipofuscin and weight nor length. This allometric relationship suggests that size of the animal was increasing at a faster rate than lipofuscin was accumulating within the body. This is comparable to Doubleday and Semmen's (2011) result for weight and lipofuscin in the optic lobes of *Octopus pallidus* where the best fitting model was also a power curve with a low R^2. They also reported that there was no relationship between body weight and lipofuscin quantity (Doubleday & Semmens, 2011) as seen in the data presented in the current study. Sheehy *et al.* (1996) also found that there was no relationship between weight or length and lipofuscin concentration. Therefore, we can hypothesize that lipofuscin concentration/quantity does not correlate with the size of the individual.

4.4.4 Accumulation of lipofuscin granules as a function of age

When using age estimates from beaks and stylets, it appeared that lipofuscin volume ratio did increase slightly with age, but the statistics showed no significant relationship between lipofuscin and age (beak and stylet age estimates). There were large fluctuations in lipofuscin volume ratio, possibly because lipofuscin formation relies on metabolic rate which can be altered due to change in temperature and individual variation (Zielinski & Portner, 2000; André *et al.*, 2009). In crustaceans, seasonal oscillations related to environmental fluctuations affect lipofuscin accumulation rate which can cause variance (Maxwell *et al.*, 2007; Harvey *et al.*, 2010).

Doubleday and Semmens (2011) is the only other published study that quantified lipofuscin quantity in an octopus species and found that it increases exponentially with age. This is comparable to the current dataset as an exponentials curve fits the data well, but a power curve fits slightly better. This suggests that the present data are similar in trend to Doubleday and Semmens (2011), but because there is so much variation, further testing should be done. Doubleday and Semmens (2011) used known-age individuals, which is what always should be done when examining an aging method for its accuracy. Maxwell *et al.* (2007) and Harvey *et al.* (2010) suggest that accumulation rate needs to be calibrated with known aged individuals under ambient environmental conditions before determination of age can be made. Once the trend is known for a given species using known-aged individuals, age can be estimated using wild caught animals fit to the trend. Known-age individuals were not used in the present study as this species is extremely difficult to rear in captivity due to their small paralarval stage. Therefore, all ages are estimates and not determinations.

Previous studies on lipofuscin in crustaceans have found a linear increase with age such as the Caribbean spiny lobster, *Panulirus argus* (Maxwell *et al.*, 2007), the crayfish, *Cherax quadricarinatus* (Sheehy, 1990a), and the krill *Euphausia pacifica* (Harvey *et al.*, 2010). In other species such as the American lobster, *Homarus americanus* (Wahle *et al.*, 1996), the relationship between lipofuscin and age is best fit by a power curve whereas in the hard clam, *Eurhomalea exalbida*

(Lomovasky *et al.*, 2002), an exponential curve is best. In the same crayfish, *Cherax quadricarinatus,* a later study found a negative exponential trend in laboratory reared individuals from 0-3 years of age (Sheehy, 1992). He attributed this trend to the slowing of metabolic rate as age increases (Sheehy, 1992). This refutes the hypothesis that lipofuscin accumulates consistently over time in this species like the previous linear trend would suggest.

When compared with lipofuscin volume values from previous studies, the values from the present study were slightly lower. Doubleday and Semmens (2011) reported lipofuscin volume ratios from 0 to 0.5% whereas ratios from the present study ranged from 0 to 0.0025%. Lipofuscin volumes in crustacean brains were also much higher from 0 to ~1.8% (Sheehy, 1990a, 1989). Lipofuscin may have been lower in this study because care was not taken to keep optic lobes from exposure to light. Because lipofuscin is a photosensitizer, light may irreversibly damage the supporters of photoreceptors, lipofuscin-loaded retinal pigment epithelial cells (Brunk & Terman, 2002; Terman & Brunk, 2004). To prevent light damage, future studies should process and store all samples in minimum light or dark to prevent fading of fluorescence.

4.4.5 Conclusions

In conclusion, these results support the hypothesis that lipofuscin can be used as a relative aging method as it increases with age and size of the individual, but a high degree of variation was found. This could be due to the uneven distribution of lipofuscin granules throughout the tissue. To prevent this in future studies, a larger number of random micrographs should be analysed such as 20-30 instead of 10. Although this method could not be used to estimate age, it was successful in identifying age cohorts as individuals from Otago were found to contain more lipofuscin granules than those from Bluff. It was also found that prepared slides would lose fluorescence quickly, so to prevent any loss of fluorescence, slides should be photographed soon after mounting.

Chapter 5: Paralarval rearing

5.1 Introduction

5.1.1 Octopus huttoni *reproduction*

Many octopus species are known to mate year round because females have the capability to store sperm in the oviducts (Wells, 1978; Cortez *et al.*, 1995). By storing sperm, the female can mate whenever she encounters a suitable male and retain the sperm until the eggs are mature. This has been observed in a range of *Octopus* spp. including *Octopus vulgaris* (Smale & Buchan, 1981; González *et al.*, 2011; Rodríguez-Rúa *et al.*, 2005; Wells, 1978; Lourenço, 2014), *Octopus tetricus* (Joll, 1976), *Octopus magnificus* (Smith *et al.*, 2006), *M. maorum* (Grubert & Wadley, 2000), *Octopus pallidus* (Leporati *et al.*, 2008a), *Octopus cyanea* (Van Heukelem, 1976), and *Octopus maya* (Van Heukelem, 1976). Mating occurs when the male inserts his hectocotylus into the mantle cavity of the female (Wells, 1978). A spermatophore (Fig. 5.1) then travels from the internal penis, up the hectocotylus arm and then discharges the sperm in the female's oviducts (Wells, 1978). In *O. vulgaris*, the sperm is stored in the oviducal glands until ready to be used (Wells, 1978).

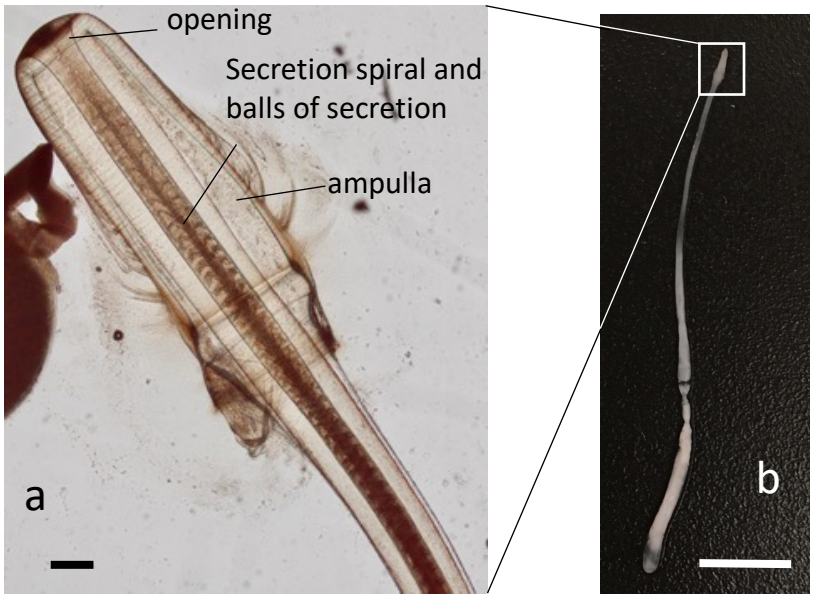

Figure 5.1. (a) Tip of an *Octopus huttoni* spermatophore. The opening, sperm secretion spiral, ball of secretion, and ampulla are indicated. Scale bar = 200 μm. (b) Full view of an *Octopus huttoni* spermatophore. Scale bar = 5 mm.

Several days or weeks prior to egg laying, many Octopodidae females may decrease feeding rates or cease entirely (Mangold, 1983; Wodinsky, 1978; Cortez *et al.*, 1995). When eggs are laid, they are typically attached to substrate in a den, but some pelagic genera are known to carry the eggs in their web (Villanueva & Norman, 2008). In either case, females care for their eggs from laying to hatching. During this time the female cleans the eggs with the suckers on her arms to avoid biofouling and gently jets water across the eggs to keep them aerated (Villanueva & Norman, 2008; Batham, 1956; Boletzky, 1994). The female is also starving because feeding would mean leaving the eggs to hunt (Villanueva & Norman, 2008; Batham, 1956; Boletzky, 1994). Without continuous attention, eggs are vulnerable to predation and susceptible to rotting due to low oxygenation and biofouling. After the eggs hatch, the female is significantly smaller and weaker and ultimately dies from starvation (Villanueva & Norman, 2008; Cortez *et al.*, 1995).

5.1.2 Octopus huttoni *eggs*

Octopus huttoni is a small egg species of octopus in that the mother produces many (~4,000-6,000 in *O. huttoni*) small eggs with less nutritional value than a large egg species (Sweeney *et al.*, 1992; Carrasco, 2014). The eggs are ovoid, measuring approximately 2.5 mm in length and 1 mm in width (Carrasco, 2014). Egg capsules are twisted together to create the chorion stalk (Fig. 5.2) which is then fixed to a hard surface, such as the top of a rock cave in the wild or a PVC tube in captivity, with a natural cement (Sweeney *et al.*, 1992; Carrasco, 2014; Villanueva & Norman, 2008; Anderson, 1999; Villanueva, 1995). In this species, the strands of eggs that are entwined make a festoon that ranges from 11 to 25.8 mm in length (Fig. 5.3) (Sweeney *et al.*, 1992; Carrasco, 2014).

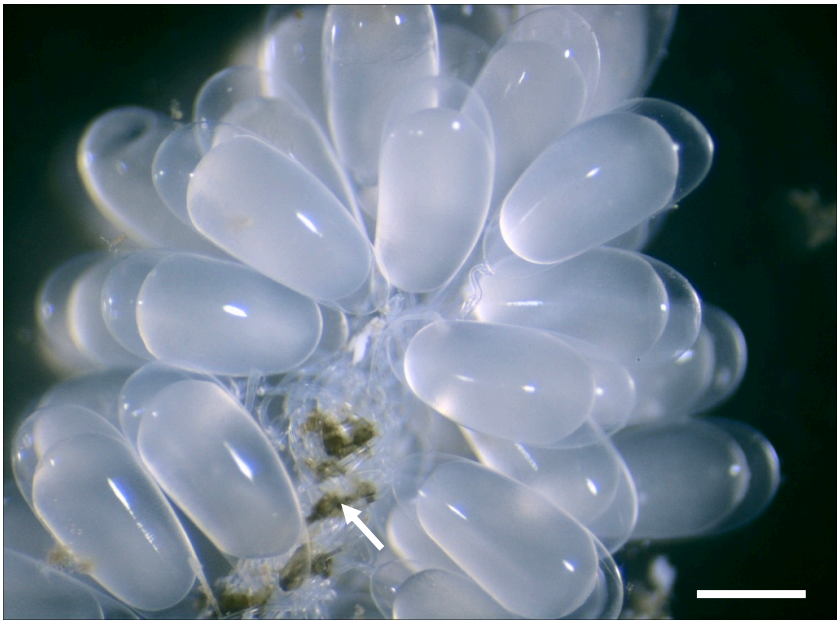

Figure 5.2. Ten-day old *Octopus huttoni* eggs incubated at a mean temperature of 15°C. The chorion stalk of and the strings that it is comprised of are indicated by the white arrow. Scale bar = 1 mm.

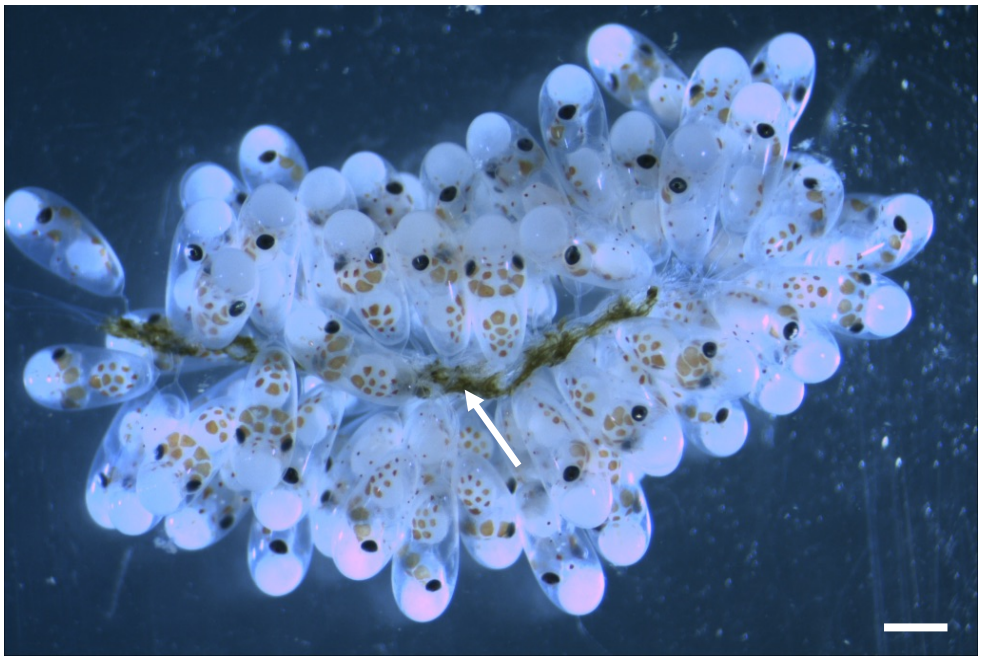

Figure 5.3. A festoon of 40-day old *Octopus huttoni* eggs incubated at a mean temperature of 17.4°C entwined to create a chorion stalk (arrow). Scale bar= 1 mm.

5.1.3 Planktonic paralarvae

Because *O. huttoni* is a small egg species, the hatchlings must develop through a free-living planktonic paralarvae stage before settling to the benthos (Carrasco,

2014). Other small egg species with slightly larger egg size have shorter planktonic phases while some large egg species (>10 mm) produce crawl-away, demersal juveniles (Sweeney *et al.*, 1992). This later strategy potentially avoids the risk of predation associated with a planktonic phase (Sweeney *et al.*, 1992; Carrasco, 2014). The hatchlings are termed "paralarvae" from the Latin prefix *"para"* meaning "almost" because they do not have distinct larval characteristics (Young & Harman, 1988) and do not undergo metamorphosis as in most species of invertebrates (Young & Harman, 1988). Planktonic paralarval duration depends on the species and environmental factors, but *O. huttoni* is thought to spend between 40 and 70 days in the water column. This is based on observations for *Robsonella fontaniana*, a morphologically similar species (Carrasco, 2014; Uriarte *et al.*, 2010).

5.1.4 Paralarval growth

A number of abiotic and biotic factors such as oxygenation and predation can influence paralarvae growth and survivability but temperature and food availability appear to have the most significant impact (Uriarte *et al.*, 2010; Vidal *et al.*, 2002; Leporati *et al.*, 2007; André *et al.*, 2008; Zúñiga *et al.*, 2013; Ramos *et al.*, 2014; Aguado Giménez & García García, 2002; Forsythe & Van Heukelem, 1987; Forsythe & Hanlon, 1988). This leads researchers to believe that octopus species with planktonic young will be greatly affected by ocean warming (Doubleday *et al.*, 2016; Higgins *et al.*, 2011).

Octopus paralarvae can only survive around 4-5 days without suitable prey (Cortez *et al.*, 1999a; Nande *et al.*, 2017). A number of studies have looked at food preferences for a variety of *Octopus* paralarvae in captivity. Preferred prey for paralarvae includes mysidacean shrimps (Derusha *et al.*, 1987; Villanueva *et al.*, 2009), *Artemia* nauplii or metanauplii (Villanueva *et al.*, 2002; Guinot *et al.*, 2013; Viciano *et al.*, 2015; Okumura *et al.*, 2005; Iglesias *et al.*, 2004; Villanueva *et al.*, 2009), decapod zoea (Villanueva, 1994, 1995; Iglesias *et al.*, 2004; Villanueva *et al.*, 2009; Villanueva & Bustamante, 2006), or non-living food such as frozen fish or millicapsules (Villanueva *et al.*, 2002; Navarro & Villanueva, 2003). Many of these prey options have been used successfully in rearing planktonic paralarvae of a

couple of species such as *O. vulgaris* (Okumura *et al.*, 2005; Villanueva *et al.*, 2002, 2009; Villanueva, 1994; Iglesias *et al.*, 2004; Guinot *et al.*, 2013; Navarro & Villanueva, 2003; Villanueva & Bustamante, 2006) and *Loligo vulgaris* (Villanueva, 1994), although each species has unique prey preferences.

If food availability is sufficient, then temperature is the most important factor facilitating growth. Perales-Raya *et al.* (2018) recently found that *O. vulgaris* paralarvae reared at 21°C were significantly heavier than those reared at 14°C, regardless of prey type. Although studies may show that paralarvae thrive in warmer water, temperatures on their outer thermal range are detrimental. Higgins *et al.* (2011) showed that when put in a tank with a thermal gradient, *O. huttoni* paralarvae avoided temperatures above 23°C. They also found that high temperatures had deleterious effects such as tissue denaturation and muscle spasms ultimately leading to death. An increase in seawater temperatures is predicted for northern New Zealand in the future which could cause the distribution of *O. huttoni* to shift poleward (Higgins *et al.*, 2011). This can also be applied to other species of merobenthic octopus because as global warming continues, a poleward shift in distribution may occur (Ramos *et al.*, 2014).

Growth of both paralarvae and juveniles needs to be investigated in addition to adults as many species are known to display two growth phases (Cortez *et al.*, 1999a). In laboratory-raised *Octopus mimus* juveniles, Cortez *et al.* (1999) found an exponential growth trend during the first 40 days of culture which then slowed and became logarithmic. Derusha *et al.* (1987) also found evidence for a two-phase growth pattern in the Pacific pygmy octopus, *Octopus digueti,* where growth was also exponential for the first 72 days after hatching and logarithmic until 143 days (end of the experiement). Villanueva (1995) and Villanueva *et al*. (2002) also reported an exponential growth trend in *O. vulgaris* paralarvae from hatching to 60-days old. If studies only examine larger individuals, they might infer that overall growth is logarithmic and may miss the shift in growth phase. Therefore, it is important to study growth during the entire lifespan of an animal from birth/hatching to death.

5.1.5 Aims

As the ocean warms, many organisms will be affected and because octopus paralarvae are sensitive to changing temperatures, studies need to be done to investigate how temperature will affect growth. This chapter aimed to determine how temperature affects the growth and survivability of *O. huttoni* paralarvae, document embryonic development, and attempt to keep paralarvae until settlement. This was done by catching females from the wild to lay and brood eggs in captivity until paralarvae hatched. Paralarvae size was also compared to female size to determine if the size of the mother determines the size and therefore health of the offspring.

5.2 Methods

Methods describing adult capture and animal husbandry are given in Chapter 2.

5.2.1 Breeding

Over the course of the study, nine pairs of *O. huttoni* from Otago and three pairs from Bluff were separated into 12 tanks for breeding. Because females can store sperm from previous mating events, it was not feasible to determine if the eggs were the product of the male placed in the tank or from previous mating. Indeed, several females laid fertilized eggs, even when they were kept in a tank alone after capture.

The ambient water temperature of the tanks (female's tanks) was recorded constantly by temperature monitors. The laying date, hatching start date, hatching end date, and the mother's death date for each clutch was recorded.

5.2.2 Documenting embryonic development

Embryonic development was documented with micrographs whenever an egg festoon was dislodged from the substrate. Eggs were never pulled away from a guarding female. Embryonic Naef stages described for *O. vulgaris* (Naef, 1928)

(Table 5.1) were used to identify different embryonic stages of *O. huttoni* as is the most similar to *O. vulgaris* in terms of reproduction (Brough, 1965).

Table 5.1. Description of Naef stages using Naef (1928) translated by Stina Kolodzey, Boletzky (1987), and Ibarra-García *et al.* (2018).

Naef stage	Description
I	1200 cell stage, blastoderm developed
I-II	Entomesoderm development begins
II-III	Entomesoderm development continues
IV-VII	Distinction of germinal disc and growth of the yolk
VII-IX	Stages of unfolding
X-XII	Primary formation of the embryo
XIII-XV	Secondary unfolding and rearrangement of the head organs
XV	End of organogenesis
XV-XVII	Development of the head, the crease, and the mantle
XVIII	Internal yolk sac refilled from outer yolk
XVIII-XIX	Developing embryos
XIX	Second reversion
XX	Hatching

5.2.3 Temperature experiment

When a female laid a clutch of eggs, all other octopus and crabs were removed from the tank to allow the female to care for her eggs without disturbance. When the majority of individuals had hatched, they were transferred to four recirculating, cylindrical kreisel tanks in a controlled temperature room. Each tank had approximatly 1,000-1,600 paralarvae in 9.6 L of seawater and 300 μm mesh over the outflow panel. The tanks were made of plexiglass with black plastic wrapped around the cylindrical portion (PML tanks) to create a background in which paralarvae can visualize their prey (Fig. 5.4). One tank was heated to 23°C, one to 17°C, and two were kept at room temperature of 11°C. These temperatures were determined from the range of sea temperatures (4.4-20°C) that are seen in the Otago Harbour near PML (Appendix C1). A 50% water change was made every day with fresh, filtered seawater which was heated or cooled to the appropriate temperature before adding to the system.

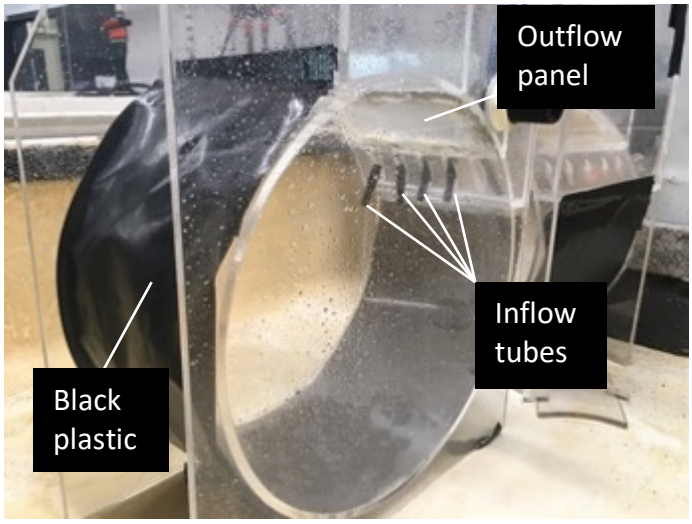

Figure 5.4. Photo of a Portobello Marine Lab tank (9.5 L) made of clear plexiglass with four inflow tubes, black plastic wrapped around the perimeter, and 300μm mesh over the outflow panel.

Paralarvae were fed freshly caught crab zoea (~1 mm in length, ~1-5 per tank) twice-daily, but as the breeding season for crabs was finishing, *Artemia* metanauplii were also used as a prey supplement. The *Artemia* were gut loaded (fed) with the red algae *Rhodamonas salina* daily and some with Docosahexaenoic acid (DHA) supplements (500:100, EPA:DHA) at the metanauplii stage 24 h before paralarvae feeding. Approximately 0.5-1 *Artemia* mL^{-1} were added to each tank once a day. Every day, ten paralarvae from each tank were sampled and three out of ten were measured for mantle length and total length by placing them in a petri dish and anaesthetised with 1% ethanol to measure growth over time. After which five were preserved in 70% ethanol and five in FAACC. The temperature of each tank was recorded twice-daily to ensure that the temperature is within 0.5°C of the targeted temperature.

When two clutches of eggs hatched at the same time, one clutch was housed in different cylindrical tanks (Zoology tanks) in the tank room at PML. These tanks were on constant flow-through, had 300 μm mesh over the outflow, held 11.6 L of seawater and approximatly 1,000-1,600 paralarvae each. The tanks were made from a slice of a large black alcathene pipe making the cylinder black and the walls clear (Fig. 5.5).

Two clutches of eggs were laid in autumn (May 2018) and when they hatched in October 2018, focus was on keeping them alive for as long as possible at ambient temperature instead of experimental temperatures. The tanks were swapped so that the Zoology tanks were in the CT room on recirculation and the PML tanks were on flow through for these last two clutches of paralarvae. This was done as an attempt to maximize survival as the Zoology tanks were found to be a better tank design. In addition to the tank change, aeration was added to the recirculated water to saturate the water with oxygen. Light was delivered at 12 h of low light and 12 h of dark switching at 8:00 h and 20:00 h. Paralarvae were fed with only *R. salina* gut loaded *Artemia* at a concentration of 0.5-1 *Artemia* mL^{-1} day^{-1}. Paralarvae found dead at the bottom of the tank were collected to check for increments on the beak (Chapter 3). A further egg clutch was laid in October 2018, but because the female needed to be euthanised for the peridocity validation experiment (Chapter 3), eggs were removed from the den and preserved in 70% ethanol.

Figure 5.5. Photo of one Zoology tank made of a black pipe with clear plexiglass sides set up with the recirculating pump in the temperature control room.

5.2.4 Analyses

Lay date and average incubation temperature were plotted against the incubation duration and fit with polynomial curves. Linear models were then run in JMP Pro (version 11.0) to test for correlation. The female's age and mantle length were also plotted against the average paralarvae mantle length at hatching for Otago and Bluff females and an ANOVA was run. Paralarvae mantle lengths with standard error were also plotted against age for a variety of different clutches to show change in length over time/age.

5.3 Results

A total of 15 out of 18 females (83%) kept in captivity laid eggs between November 2017 and October 2018 because the remaining three females were euthanised before they could lay. Of these females, eight were able to successfully incubate their eggs until hatching, while six either died during incubation (n=3) or abandoned their eggs (n=3). Incubation period ranged from 43-158 days and although there was premature hatching (1-20 paralarvae day^{-1}), bulk hatching (>1,000 paralarvae day^{-1}) lasted for approximately two days on average. After hatching, the females stayed alive for 7-42 days before dying.

Females were noticeably thinner due to starvation and depositing their eggs (Fig. 5.6a). Their skin was almost transparent, their bodies were jelly-like (Fig. 5.6a) and smelled as if they were rotting. Pupils also looked circular (as opposed to the normal horizontal slit (Fig. 5.6d)) and took up almost the entire eyeball (Fig. 5.6c).

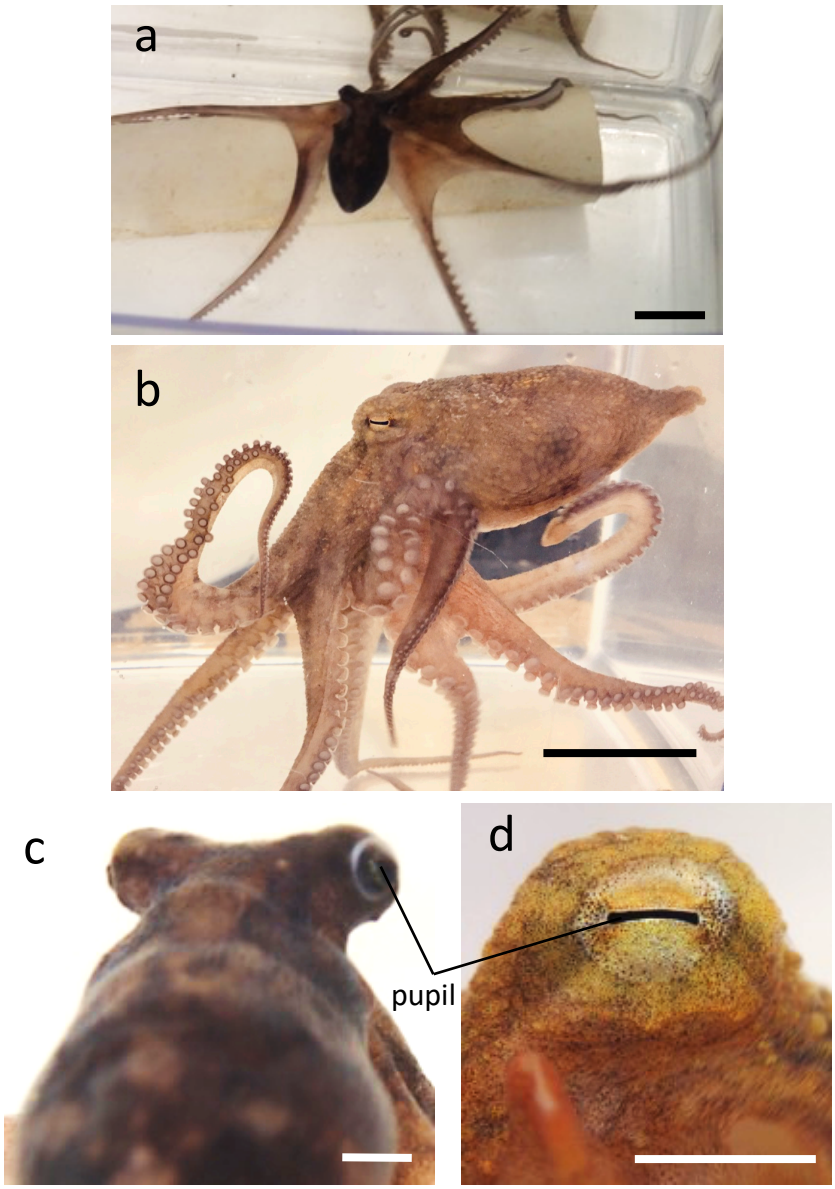

Figure 5.6. Photos of *Octopus huttoni* showing a spent female (a), healthy male (b), the protruding eye of a spent female (c), and the eye of a non-senescent adult (d). Scale bars = (a and b) 20 mm, (c and d) 5 mm.

Egg clutches were laid and paralarvae hatched all throughout the year (Fig. 5.7). There was no correlation between paralarvae mantle length at hatching and the female's age at death nor female's mantle length at death (Fig. 5.8).

Figure 5.7. Water temperature (°C) on the dates when *Octopus huttoni* eggs were laid (orange square) and paralarvae bulk hatched (green triangle).

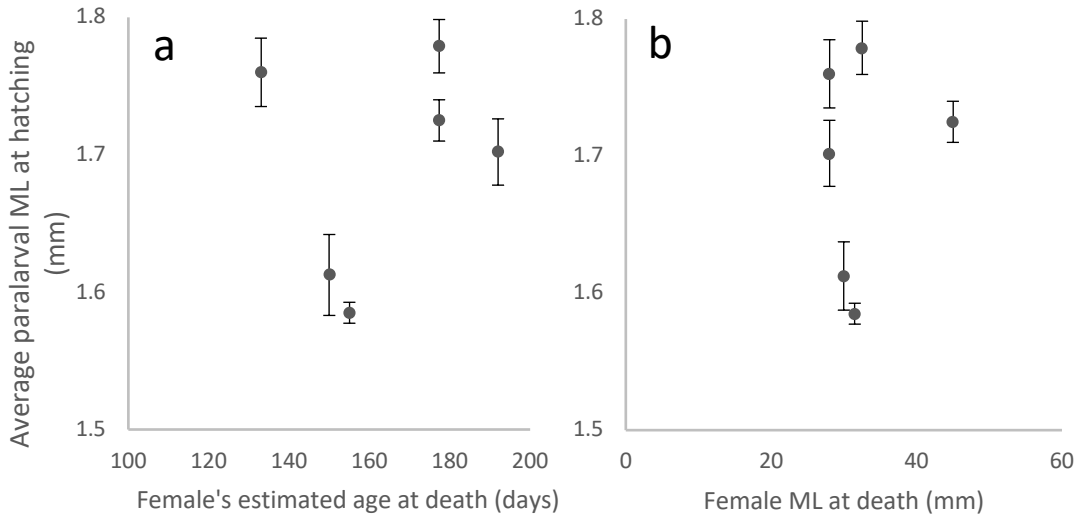

Figure 5.8. Comparison of *Octopus huttoni* average paralarvae mantle length (ML) at hatching with the female's estimated age (days) (a) and mantle length (ML)(b) at death (n=6). Error bars are standard error.

Incubation period of egg clutches depended on the time of the year they were laid; increasing from November (Summer) to May (Winter) (Fig. 5.9). This correlation was proven significant by a linear model ($F_{2,8}$=73.596, p<0.0001, R^2=0.91). This is represented by temperature as incubation period decreased with increasing water

temperature (Fig. 5.9). This correlation was also proven significant (Linear model, $F_{2,8}$=2.51.835, p<0.0001, R^2=0.97).

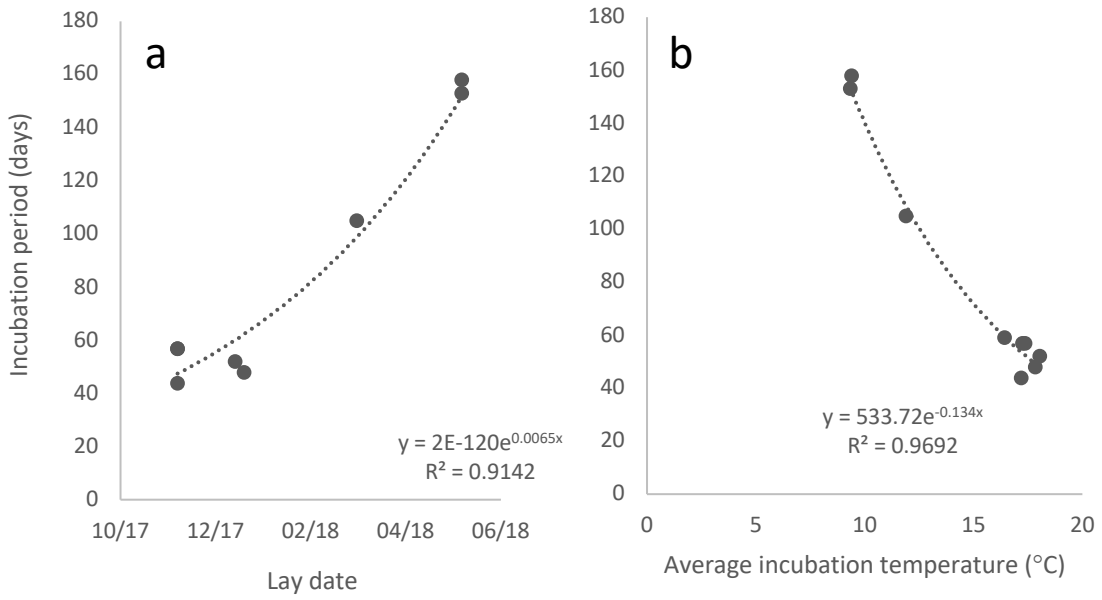

Figure 5.9. Incubation period (days) of *Octopus huttoni* eggs with lay date (a) and average seawater temperature (b) (°C) during incubation (n=8). Fit with exponential curves.

Egg size was on average 2.3 ± 0.2 mm in length and 1 ± 0.1 mm in width (Fig. 5.10). Number of eggs laid per female ranged from ~3,800 to 6,500 with a range of ~50-125 eggs per festoon/egg bunch.

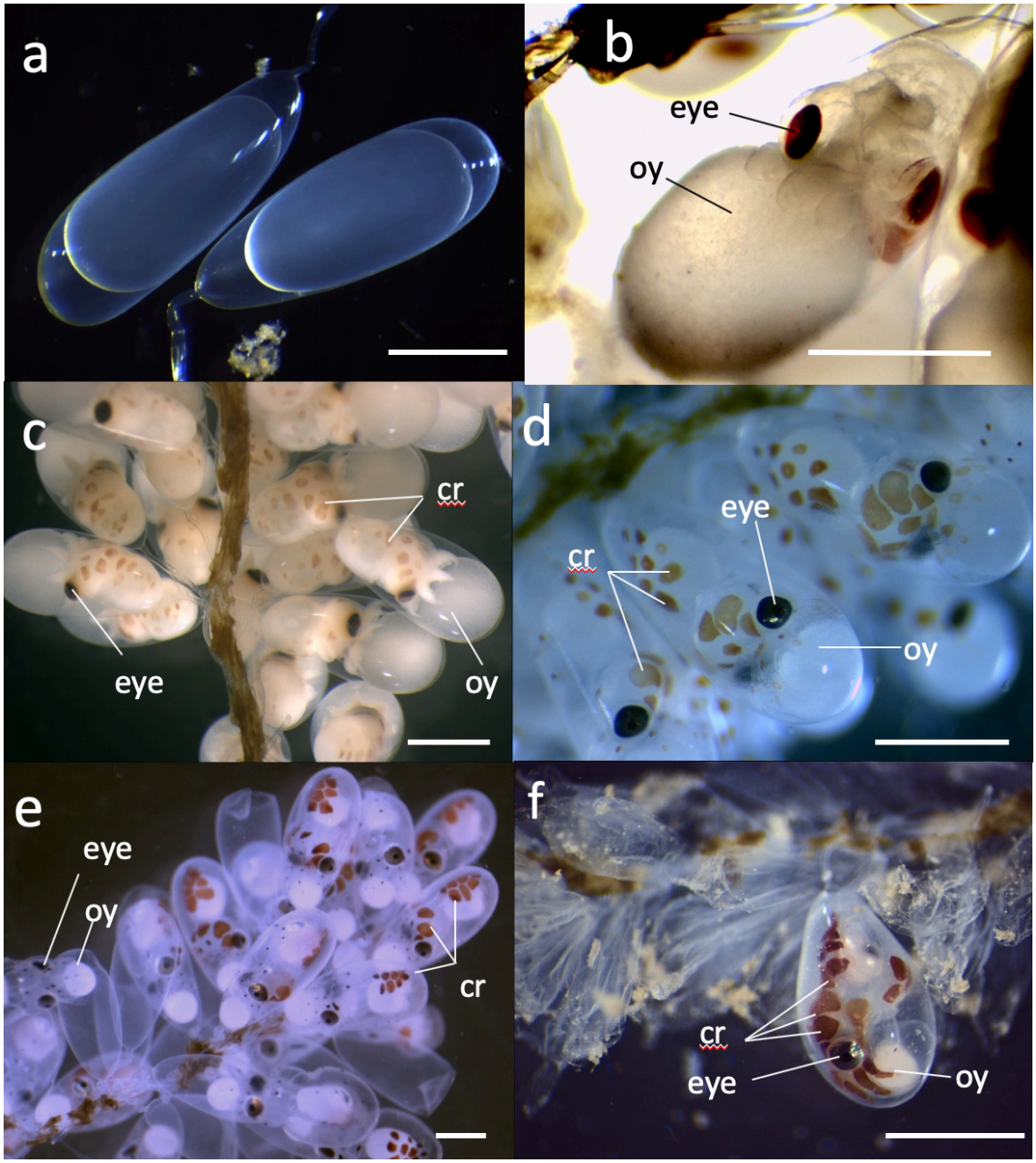

Figure 5.10. Embryonic development of *Octopus huttoni* eggs staged using Naef stages of development (Naef, 1928). Naef stage I: 17 days after laying at an average temperature of 9.5°C (a). Naef stage XIII: at the end of organogenesis with the development of eyes, 97 days after laying at an average temperature of 8.8°C (b). Naef stage XVI: post-organogenesis with the development of chromatophores (micrograph taken after preservation in 70% ethanol), 32 days after laying at an average temperature of 17.8°C (c). Naef stage XIII: 40 days after laying at an average temperature of 17.2°C (d). Naef stage XX: (hatching) showing second rotation within the capsule 47 days after laying at an average temperature of 16.3°C (e). Naef stage XX: paralarvae that did not turn itself before growing larger and was stuck in the egg while others hatched 107 days after laying at an average temperature of 11.8°C (f). cr: chromatophores, oy: outer yolk. Scale bars = 1 mm.

Cannibalism was observed in paralarvae as well as hunting of crab zoea. Hunting of *Artemia* was never observed, but all sampled individuals had pink guts, which was assumed to be from the pink *R. salina*-rich *Artemia* (Fig. 5.11b).

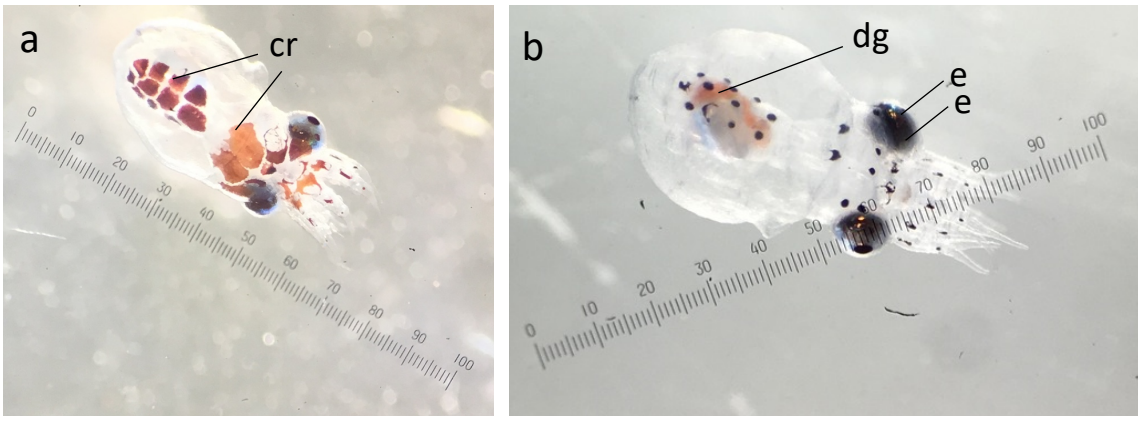

Figure 5.11. Photos of *Octopus huttoni* paralarvae from the dorsal side (a) and ventral side (b) viewed at 2x magnification. dg: digestive gland, cr: chromatophore, e: eye.

The duration of paralarval survival ranged from 3 to 19 days after hatching. Clutches reared at ambient temperature survived for 3, 7, and 8 days and paralarvae mantle length (mm) showed a slightly declining growth trend (Fig. 5.12).

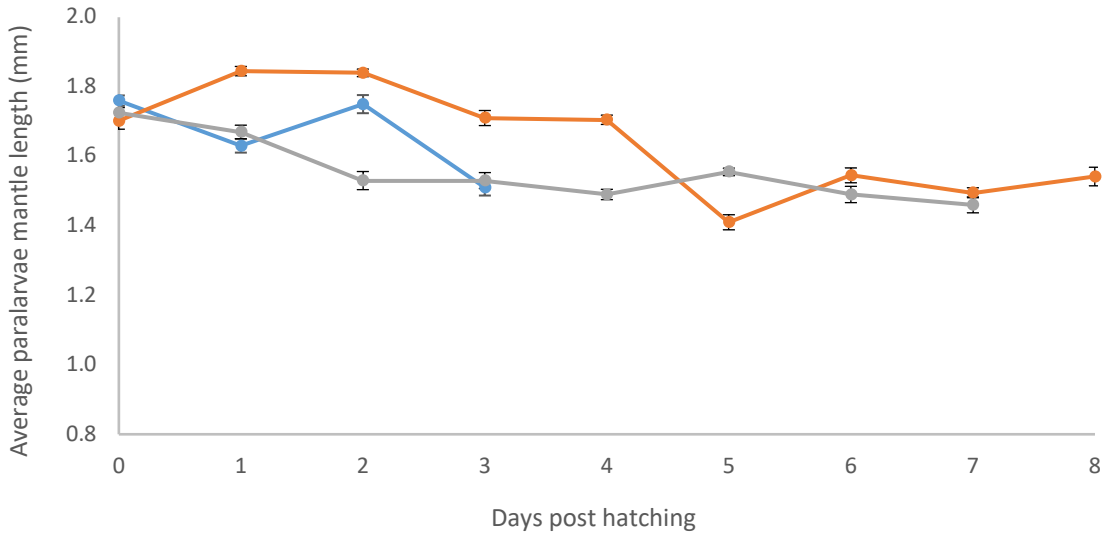

Figure 5.12. Average mantle length (mm) of *Octopus huttoni* paralarvae kept at ambient temperature (17.6-18.4°C) for the first 8 days post-hatching where the different colours represent paralarvae from different females. Error bars are standard error.

Clutches reared in the temperature experiment survived for 5 and 7 days, during which time mantle length showed a slight decline in size (Fig. 5.13). The longest that paralarvae from a single clutched survived was 19 days, which were reared at ambient temperatures in recirculating tanks. A second clutch survived for 15 days, also reared at ambient temperature in flow-through tanks. Focus was on keeping paralarvae alive therefore no size data were recorded.

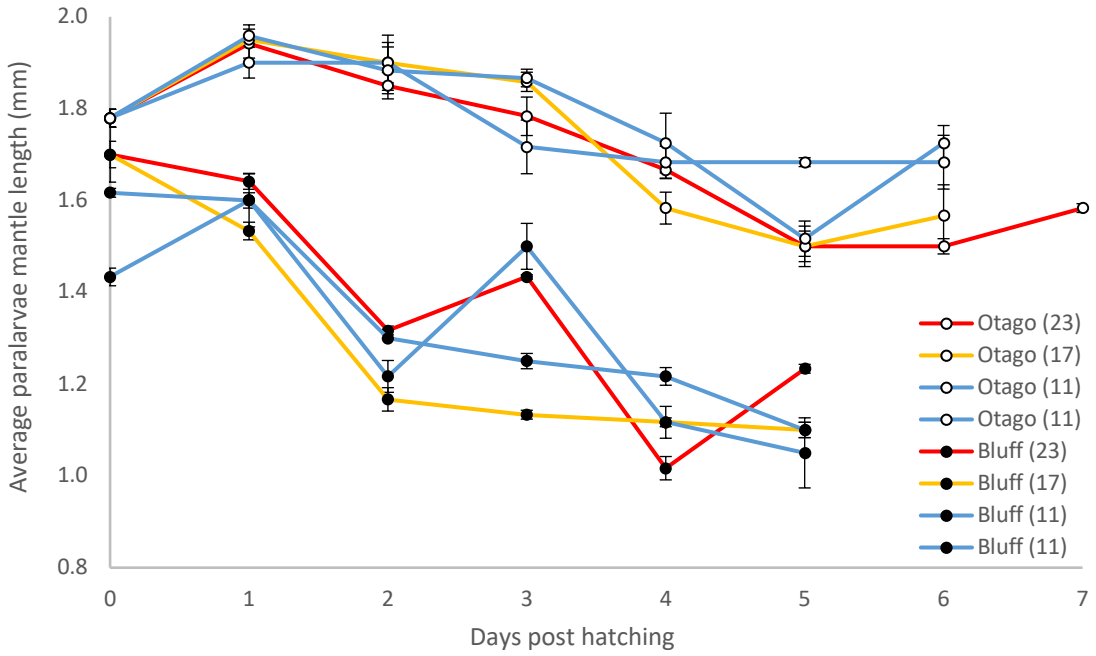

Figure 5.13. Average mantle length (mm) of *Octopus huttoni* paralarvae from a Bluff female and an Otago female reared at different temperatures (23, 17, and two replicates at 11°C) for the first 5- and 7-days post-hatching. Error bars are standard error.

5.4 Discussion

5.4.1 Laying and brooding eggs in captivity

Almost all females kept in captivity (83%) laid eggs and 60% of females successfully brooded, and hatched paralarvae. Abandoned eggs were often eaten by the mother or ignored until they rotted in the tank. This could have either been due to stress on the mother or the eggs being unviable. When eggs were abandoned, the mother would still not take food, but instead appeared stressed and walked aimlessly around the tank. These are symptoms of a senescent female that has laid infertile eggs (Anderson *et al.*, 2001).

Spent, senescent females that had ceased feeding appeared malnourished with thin skin. As they starve, senescent females have high metabolic rates as they utilise the dissolution of muscle tissue as a fuel source (Moltschaniwskyj, 1995; O'Dor & Wells, 1978). This causes the body to shrink, especially the mantle. Just before the females died they smelled as if they were rotting possibly because they were deteriorating just like senescent *Octopus briareus* (Hanlon, 1983). Their pupils also appeared dilated. This is most likely due to the retraction of skin around the eye which occurs during senescence, thus making the eyes more visible and protruded (Anderson *et al.*, 2001). One symptom in senescent males is undirected and uncoordinated activity in which they walk around clumsily (Anderson *et al.*, 2001). Unlike senescent males, spent females would not leave their dens, even after the eggs had hatched and strongly resisted being prodded out as if they were still guarding eggs.

Females were observed to lay eggs all year-round regardless of temperature. Paralarvae also hatched at a range of temperatures, indicating that temperature may not be the cue to lay eggs nor for paralarval hatching. Hatching normally occurs as soon as the outer yolk sac is exhausted, however the paralarvae in the current study typically hatched with some yolk. Premature hatching is typically associated with a loss of nutrients because paralarvae drop the yolk sac after hatching, but some species can absorb it post-hatching (Boletzky, 2003). Hatching before yolk is completely used is only advantageous if the microclimate in the egg is degrading due to extreme temperature changes, lack of oxygen, or pollution (Boletzky, 2003). It could not be determined if these larvae were hatching early and if it was detrimental to survival, but yolk sacs were never found so it is assumed that most yolk was absorbed.

There was no correlation between paralarvae size and mother's estimated age nor size. This may be due to the fact that octopus are semelparous animals and only lay eggs once in their life span. Therefore, if the animal is mature and environmental cues indicate that it is time to spawn the animal will spawn regardless of age or size. Leporati *et al.* (2008) also report that age and size do not determine maturity in *Octopus pallidus* and that the cue to mature and spawn is most likely season

dependant. Forsythe and Hanlon (1988) and Cortez *et al.* (1995) also suggest that reproduction is driven by seasons, specifically temperature.

5.4.2 Embryonic development

Incubation period decreased with increasing temperature, indicating that temperature largely affects the time for embryonic development. This is consistent with what is known about embryonic development in cephalopods. Many different species of squid and octopus such as *O. mimus* (Uriarte *et al.*, 2012), *Sepia officinalis, O. vulgaris, Sepiola robusta, L. vulgaris, Loligo pealei*, and *Illex illecebrosus* (Boletzky, 1994) have been shown to have increased embryonic development periods when temperatures are lower. Egg size also affects development time, with smaller eggs developing quicker than larger eggs incubated at the same temperature (Boletzky, 1994). Within a species, temperature is an important factor in determining development time. In the squid *Sepiola,* eggs developed slower (2.5 months) at 13°C than at 15°C (2 months) (Boletzky, 1983). *O. mimus* reared at 21°C also reached stage XV 24, 58, and 75% faster than at 18, 15, and 12°C, respectively (Uriarte *et al.*, 2012). It is unknown how this difference in temperature and development time affects the health or survivability of the paralarvae. In the current study, the two clutches which were incubated for 153 and 158 days, survived to 19- and 15-days post-hatching, respectively. It cannot be determined if this increase in survivability was due to a longer incubation time or modification of tank set up, so further studies are needed to be certain.

Embryonic development did not seem to differ from that observed in other species of octopus such as *Hapalochlaena sp.* (Overath & von Boletzky, 1974)*, O. mimus* (Uriarte *et al.*, 2012)*,* and *O. vulgaris* (Naef, 1928) and was consistent with other studies on *O. huttoni* (Brough, 1965; Carrasco, 2014). Egg size was also consistent with the other studies that observed embryonic development in *O. huttoni* (c.f. Carrasco, 2014: length = 2.47 mm and width = 1.2 mm; Brough, 1965: length = 2.5-2.9 mm and width = 1-1.2 mm). Total number of eggs and number of eggs per festoon is also consistent with previous work (Carrasco, 2014: ~4,600 eggs and ~75 eggs per festoon).

Paralarvae always hatched with the outer yolk, which was assumed to be used up as yolk sacs were never found in the tank. Normally this would indicate that the paralarvae hatched prematurely and while this did occur, paralarvae also hatched with yolk during bulk hatches (>1,000 day^{-1}). The presence of the outer yolk sac is often seen when reared under artificial conditions and could possibly affect their survivability if they drop the yolk instead of first absorbing it (Overath & von Boletzky, 1974; Boletzky, 1987). At this time, it cannot be determined if paralarvae were hatching prematurely and if that ultimately decreased their survivability, but the evidence presented suggests that this is true.

5.4.3 Rearing planktonic paralarvae in captivity

Clutches reared at ambient temperature and controlled temperatures did not show any indication of growth in mantle length up to 8 days. It is possible that *O. huttoni* has a non-growth period post-hatching, as seen in *O. maya.* Moguel *et al.* (2010) and Rosas *et al.* (2014) previously showed that *O. maya* has a non-growth phase of 10- and 15-days post hatching, respectively. Paralarvae that were able to survive until 19 days in the present study were not sampled for length measurements so it cannot be determined if growth began after 10 or 15 days. Brough (1965) successfully kept one paralarva up to three weeks, but no size/growth data is available to determine if there was a non-growth phase. Another hypothesis could be that paralarvae from this study were increasing in weight but not length. To test this, future studies should measure weight as well as length.

During the temperature rearing experiment, paralarvae did not survive longer than 7 days. This could be attributed to a variety of factors other than temperature because there was no difference in growth rate between temperatures. It is possible that tank design is the most important factor in paralarval rearing in captivity. If the flow was too high, the paralarvae would be hit by the water streams and scraped across the walls, causing injury to their arm tips and skin. If the flow was too low, paralarvae would settle to the bottom and die. Many of the dead paralarvae that were sampled were missing the tips of their arms or had a tear in the mantle. This damage could also be due to the high density of paralarvae

per tank (100 paralarvae per L) as there is more of a risk of swimming into each other. Sánchez *et al.* (2013) successfully reared *O. vulgaris* at a density of five paralarvae L^{-1} and Perales-Raya *et al*. (2018) use six paralarvae L^{-1}. Future studies should use much larger tanks such as Sánchez *et al.* (2013) suggests thereby minimising contact with surfaces.

The type of inflow and the angle of the water stream is also important for paralarval rearing. A spray bar pointed down against the wall is suggested as the smaller streams of water should not damage the paralarvae skin as much as the large streams. The smaller holes on the spray bar should be placed close together as to not produce any areas without constant flow as paralarvae can congregate there and die. In terms of flow, as long as it is constant throughout the tank without dead areas and not too strong, paralarvae should be well aerated.

Because the density of paralarvae in the tanks was so high (100-200 paralarvae L^{-1}) and the paralarvae were actively swimming as plankton, an aeration system is suggested for recirculating systems. The change to a tank with a spray bar and bubbler allowed the maintenance of paralarvae up to 19 days at ambient temperature instead of the 7 days from the temperature experiment. With the spray bar, there was a problem with the outputs getting clogged so that areas without flow would occur. In the future, slightly larger holes (1 mm) should be drilled in the spray bar to prevent easy clogging.

Density of prey also needs to be monitored because it was found that if too many prey (1 *Artemia* mL^{-1}) was added, there was a high risk of the paralarvae choking on the prey. Occasionally, a paralarva would be swimming erratically or bursting across the bottom of the tank and when viewed under the microscope, it would have prey stuck in its siphon. It would then try and push it out by flushing its siphon and curl its arms up to try and pull it out. Another way to avoid this problem is to use prey slightly larger than *Artemia* nauplii (1 mm). This also applies to any small debris in the tank. All debris should be cleaned out of tanks daily to avoid this problem and minimize objects that could become projectiles and damage paralarval skin. At times, prey was added in much smaller quantities or not at all for one day if there was still a significant number of prey left in the tank

in order to avoid over-crowding. These modifications in tank design and rearing set up will aid future research that may be done on *O. huttoni* rearing.

5.4.4 Conclusions

In conclusion, egg laying, brooding, and embryonic development stages were consistent with what was reported in other octopus studies where incubation time increased as temperature decreased. This indicates that at colder temperatures, eggs take longer to develop. Rearing experiments were not successful in observing growth because length slightly declined over time and weight and width of paralarvae were not measured. This could either be attributed to some factor inhibiting growth or this species could have a non-growth phase such as in other octopus species. Tank design was discussed, and it was determined that paralarvae should be reared in larger tanks at lower densities. Overall, this study provides information that is useful for future studies on octopus paralarval rearing in captivity.

Chapter 6: General Discussion

This thesis aimed to (1) investigate size, age, and condition differences between different populations and datasets, (2) compare different aging methods to determine validity, (3) model size at age, (4) document embryonic development of octopus eggs, and (5) observe the effect temperature has on the survivability and growth of octopus paralarvae. Conclusions pertaining to each aim are discussed further.

6.1 Size and age differences between different populations and datasets

Octopus huttoni caught in the Foveaux Strait and offshore of Otago were smaller and younger than those caught in the Otago Harbour. These results support the ontogenetic shift hypothesis in which planktonic paralarvae of merobenthic octopus are pushed offshore by currents, settle to the benthos, and then migrate inshore to spawn when mature. This hypothesis is supported by size and age estimates collected by beak increment analysis, stylet increment analysis, upper beak length, stylet weight, and lipofuscin volume ratio. Previous works also support this hypothesis as Stranks (1996) suggested that species with planktonic stages could be transported offshore by coastal currents. González *et al*. (2011) and Mayo-Hernádez *et al.* (2013) also show evidence in favour of this hypothesis as they suggest that adults of the merobenthic species *Octopus vulgaris* migrate inshore to spawn. They also found immature individuals to predominate the depths offshore (González *et al.*, 2011; Mayo-Hernández *et al.*, 2013) such as was found in the current study.

Individuals caught in 2017-2018 were overall larger than those caught in 2004-2006, possibly because of an increase in temperature. Sea surface temperature has increased 0.10°C decade^{-1} in the past 50 years in Southern New Zealand (Shears & Bowen, 2017). Some species of octopus are known to grow larger at warmer temperatures such as *O. vulgaris* (Smale & Buchan, 1981), which could be the case for *O. huttoni*. Although individuals caught from 2017-2018 were larger, there was more of an isometric relationship in weight and length suggesting a loss of

condition. This could be explained by the increased prevalence of parasites as four different kinds of parasites were found in recently caught animals and none were found in the 2004-2006 individuals. Mackenzie *et al.* (2014) suggest that the combined effects of increased temperature and decrease in pH may increase the abundance and diversity of parasites as was seen in the mussel, *Mytilus edulis*. These results suggest that parasites could be thriving with ocean warming and affecting host condition.

6.2 Applicability and comparison of aging methods

Beak increment analysis was found to be the best aging method, as age estimates (increment counts) were highly correlated with the size of the individuals. Incrementation of the beaks could not be validated as no increments were found on paralarval beaks, but they are predicted to be deposited daily such as *in O. vulgaris* (Perales-Raya *et al.*, 2014a) and *Octopus maya* (Rodríguez-Domínguez *et al.*, 2013) . This could be because beaks may not deposit increments until later in life such as settlement. Daily deposition of growth rings on the stylet was validated with tetracycline staining in adults where food was abundant, but because the nucleus was difficult to visualise, stylets were likely an underestimate of age. Stylets of the merobenthic octopus, *Macroctopus maorum,* were also difficult to read in that the nucleus was not clearly visible and the first increment could not be determined (Doubleday *et al.*, 2011). Doubleday et al. (2011) attributed this due to the soft nature of the stylets and predict that stylets in merobenthic species with planktonic young do not form until sometime post-hatch.

Lipofuscin increased with age and size of the individual supporting the hypothesis that lipofuscin increases with age. There was a high degree of variation in the data, which could be due to the uneven distribution of lipofuscin granules within the optic lobe because only 10 random micrographs were analysed. Maxwell *et al.* (2007) and Sheehy (2002a) suggest analysing >20 micrographs because of the uneven distribution on lipofuscin granules in eye stalks of the lobsters *Panulirus argus* and *Homarus gammarus*. Because known-aged individuals could not be used and there was a high degree of variation, lipofuscin could not be used as a direct

aging method. However, lipofuscin can be used to identify age cohorts, which was successful. Otago individuals had significantly higher amounts of lipofuscin than Bluff individuals, further supporting the suggestion that Otago individuals are older than those from Bluff.

6.3 Growth modelling

Both the Richard's curve and von Bertalanffy growth model fit the data well, but the Richard's curve predicted a more realistic maximum size (L_∞=58.76 mm) than the von Bertalanffy (L_∞=110.4 mm). This suggests that *O. huttoni* displays growth that slows at the end of the life cycle like most species of cephalopods whereas others display exponential growth in which there is no slowing of growth (Moltschaniwskyj, 2004).

6.4 Embryonic development

Egg laying, brooding, and embryonic development of *O. huttoni* were consistent with what was reported for *O. huttoni* (Brough, 1965; Carrasco, 2014). It was found that at colder temperatures, eggs took longer to develop. This phenomenon was also reported in a variety of cephalopods such as *O. mimus* (Uriarte *et al.*, 2012), *Sepia officinalis, O. vulgaris, Sepiola robusta, L. vulgaris, Loligo pealei*, and *Illex illecebrosus* (Boletzky, 1994).

Eggs were laid and hatched all year-round, which supports the hypothesis of year-round reproduction. Settlement date calculated from estimated beak ages also showed year-round settlement with a peak in spring (September and October). This is assuming that increments on beaks do not form until settlement. Other species of octopus also have peak in spawning and recruitment during spring/summer such as *M. maorum* (Anderson, 1999), *Octopus cyanea* (Herwig *et al.*, 2012), and *Octopus magnificus* (Smith *et al.*, 2006). It has been reported that southern calamary, *Sepioteuthis australis*, paralarvae gain weight faster when hatched in the spring/ summer than winter/autumn (Pecl, 2004). This is most

likely because during spring, rising temperature and prey availability therefore increases juvenile growth rate and survivability (Pecl, 2004).

6.5 Effect of temperature on paralarval growth

Paralarval mantle length showed no indication of growth up to eight days of life whether reared at ambient temperatures in flow-through systems or in temperature-controlled recirculating systems. This could be due to some factor inhibiting growth such as improper tank design and flow or this species could have an early non-growth phase such as *O. maya* (Moguel *et al.*, 2010; Rosas *et al.*, 2014). Because no growth was observed, it could not be determined if temperature was affecting paralarval growth. All other factors such as tank design and density of animals would need to be optimised for paralarval survival before it can be determined that temperature is the factor affecting growth. Sánchez *et al.* (2013) and Perales-Raya *et al.* (2018) have successfully reared *O. vulgaris* at densities of five paralarvae L^{-1} and six paralarvae L^{-1} respectively. Perales-Raya *et al.* (2018) used 500 L tanks and Sánchez *et al.* (2013) found that *O. vulgaris* paralarvae growth faster in larger tanks. Therefore, large tanks with low densities of animals is optimum.

6.6 Suggestions for future research

As this kind of research has never been done on this species of octopus, many suggestions should be considered before this study can be repeated. To fully understand and compare the habitats in which these animals live in at each site, environmental data such as temperature at depth and biological data such as prey type should be collected at time of capture.

In order to identify the age of first beak increment formation, paralarvae should be reared at least until settlement. This is difficult because small egg species are notoriously difficult to rear in captivity, but the use of much larger rearing tanks (at least >12 L) with a lower density of animals (at least <100 L^{-1}) should increase survival. Paralarval weight should also be recorded in addition to length to further

investigate paralarvae growth as weight may have been increasing in this study but not length.

To further test lipofuscin as an aging method, known-age individuals should be used as a baseline, but because this species has never been reared from hatching to settlement, successful rearing must first be done. In addition, more photos (20-30) per sample should be analysed for lipofuscin volume because of the uneven distribution of lipofuscin granules throughout the optic lobe. Lastly, because of the photosensitive nature of lipofuscin, slides should be photographed soon after preparation to avoid loss of fluorescence.

6.7 Conclusions

In conclusion, evidence that supports the ontogenetic shift in habitat hypothesis was presented in this study as *O. huttoni* caught offshore were smaller and younger than those caught in the Otago Harbour. This result was concluded using beak increment analysis, stylet increment analysis, beak length, stylet weight, and lipofuscin volume ratio indicating that these methods can be used to identify different cohorts.

It was learned that temperature is an important factor in determining the duration of embryonic development as lower temperatures slow development. Egg-care and embryonic development observed in this study was consistent with what was observed previously for *O. huttoni* and *O. vulgaris,* a reproductively similar species. Evidence of year-round reproduction with a peak in spring was presented in this study as captive females spawned year-round and estimates of settlement date also showed year-round settlement with a peak in spring.

Environmental factors such as tank design and saturated oxygenation proved to be more important for paralarval survival in captivity and paralarvae size than biotic factors such as the size and age of the mothers. Overall, this study provided crucial information on the life history of the midget octopus, *O. huttoni* and can be used as a baseline for future studies.

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Appendix

Appendix A. Temperature variation

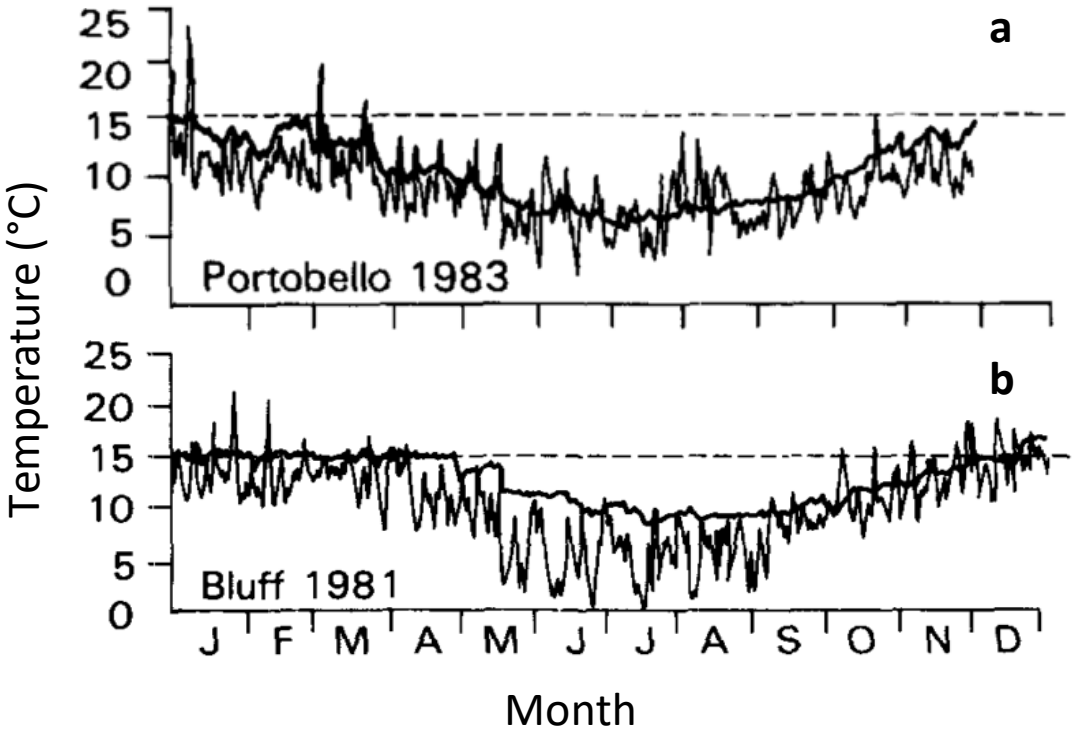

Figure A1. Variation of sea surface temperature (SST °C) throughout the year at (a) Portobello and (b) Bluff Harbour in 1983 and 1981 respectively with a dotted line through 15°C for reference (Adapted from Greig et al., 1988).

Appendix B. The discovery of parasites

Introduction

Parasites occur in many species of cephalopod all over the world, which is not surprising given the large number of parasitic species and the important role that cephalopods play in the trophic food web (Pascual *et al.*, 2007). A coccidian parasite called *Aggregata* is one of the most studied parasites in cephalopods because of its prevalence in the common octopus, *Octopus vulgaris.* It is known to cause malabsorption syndrome and decrease plasma proteins, which can then cause further damage to the infected area in the form of tissue hypoxia, necrosis, and atrophy (Gestal *et al.*, 2007). This is a result of the deceasing oxygen carrying capacity of the limited amount of plasma proteins (Gestal *et al.*, 2007). In addition to malabsorption syndrome, these changes could negatively affect growth, development, and condition of the individual (Gestal *et al.*, 2007; Pascual *et al.*, 2007; Gestal *et al.*, 1999; Pascual *et al.*, 2010). Therefore, presence of parasites is important to note when measuring growth and condition.

Methods

During dissections, parasites were found throughout the body. One that was identified as a coccidian was observed by sampling infected tissue. Skin infected with the coccidian was collected and prepared by histological methods in an attempt to identify the parasite to species. This was done by preserving the skin in Davidson fixative for 48 h at room temperature and then placed in 70% ethanol until processing. Davidson fixative was made with 100 mL of glacial acetic acid, 300 mL of 95% ethanol, 200mL of 10% formalin, and 300 mL distilled water. The tissue was then dehydrated in 95% ethanol for one hour and 100% ethanol for two washes of one hour. The tissue was then cleared with xylene twice for 40 minutes each and embedded in paraffin wax for three sets of 40 minutes with a wax change in between each set. The embedded tissue was then set in a paraffin block to cool overnight and sectioned with a microtome (Leica RM2125RT) to create 5μm sections, which were placed on a slide using a cold (16°C) and warm (45°C) water bath. The slides were de-waxed in xylene, rehydrated with an ethanol series

leading up to water, stained with hematoxylin, rinsed in water, stained with eosin, dehydrated in another ethanol series leading to xylene, and then mounted with a coverslip using Entellan®. The slides were then observed under microscopy and photos were taken as references in an attempt to identify the coccidian.

Results

Four different kinds of parasites were found in *O. huttoni* mantles over the course of this study; coccidian parasites (Fig. B1), larval cestodes (Fig. B2), trematodes (Fig. B3), and a nematode (Fig. B4). Overall, 7% of *O. huttoni* were infected with the coccidian parasite, 6% were infected with larval cestodes, 7% were infected with a trematode, and 1% was infected with a nematode (Table B1). The coccidian parasite was usually found in the connecting membrane surrounding the organs (heavily infected) (Fig. B1) and sometimes found in the stomach or spiral caecum. The trematodes were typically found in the renal sac or in the surrounding membrane (Fig. B3). The nematode which was found in one individual was discovered spiralled within the inner mantle muscle (Fig. B4). It was buried deep into the muscle and could not be removed for identification. The larval cestodes were commonly found in the intestine or stomach in which they were either enclosed in their cyst or bursting out (Fig. B2). Almost all of the infected individuals were found dead in their tanks after a few weeks of not eating. Those found heavily infected with the coccidian parasite had very jelly-like bodies and smelled much stronger than others.

Table B1. Occurrence of parasites in *Octopus huttoni* caught in Otago and Bluff

Parasite	**Otago**	**Bluff**	**Total**
Coccidian	1 male	6 males, 1 female	8 (7%)
Trematode	1 male, 2 females	2 males, 3 females	8 (7%)
Nematode	1 male	none	1 (1%)
Larval Cestodes	6 males	1 male	7 (6%)

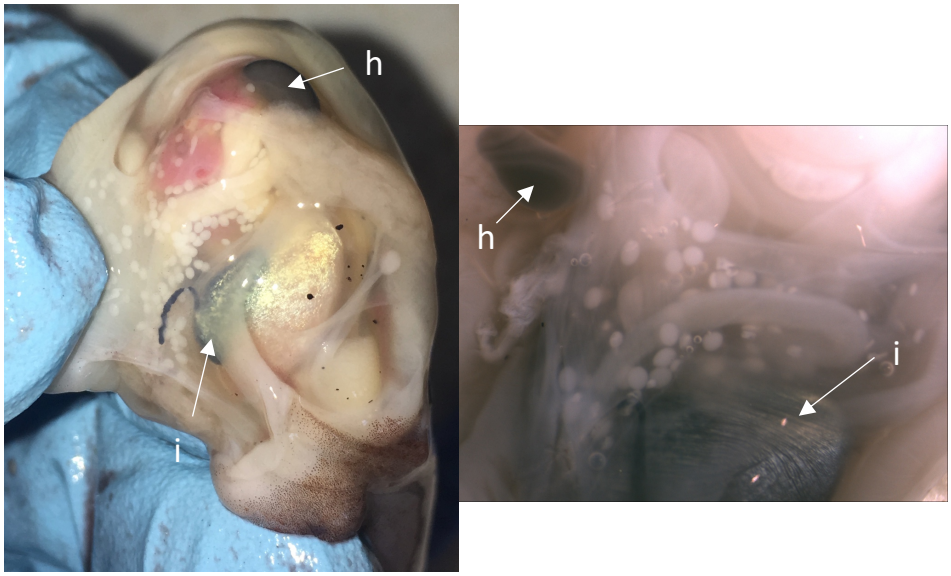

Figure B1. Coccidian parasites (white spots) in the connecting membrane in the mantle of a heavily infected male *Octopus huttoni* where the white arrows indicate the heart (h) and ink sac (is) for reference. (a) shows the appearance of the parasites from the naked eye and (b) shows the parasites under a dissecting microscope (2x).

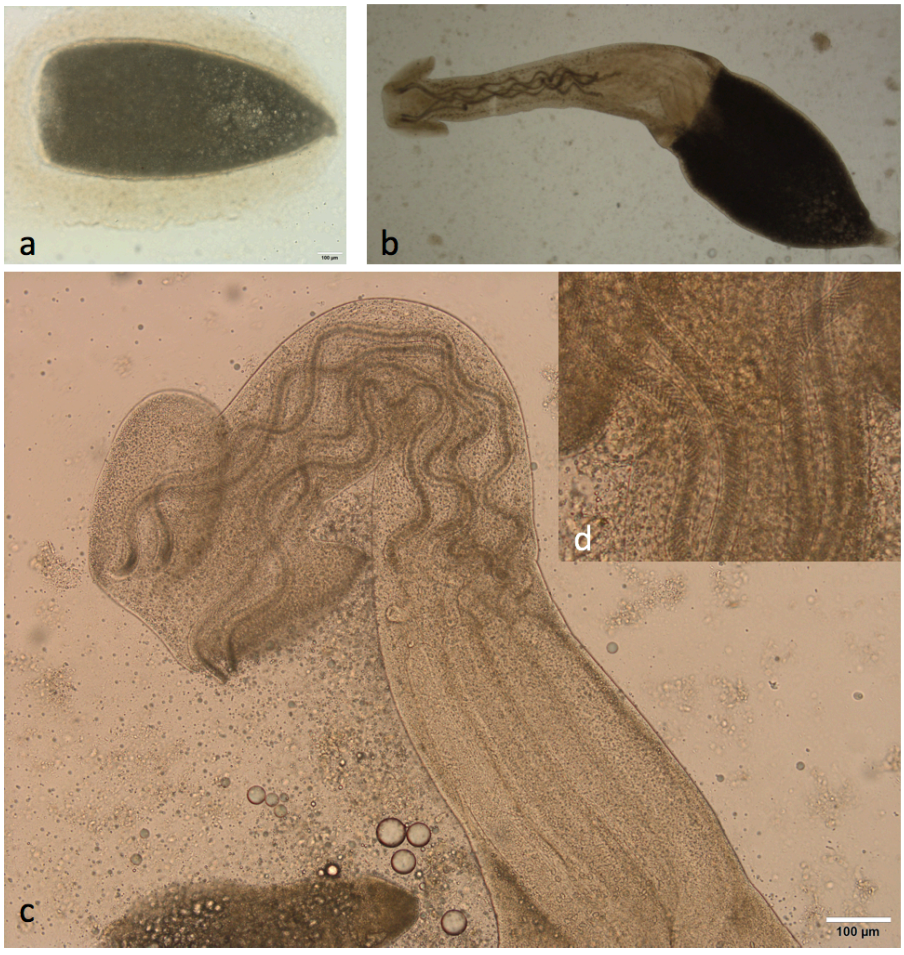

Figure B2. Photos of cestode larvae found in the digestive tract of *Octopus huttoni*. (a) Cestode cyst, (b) larval cestode emerging from cyst, (c) closer view of the larvae, (d) closer view of the four tentacles with hooks (t). Scale bars = 100 µm.

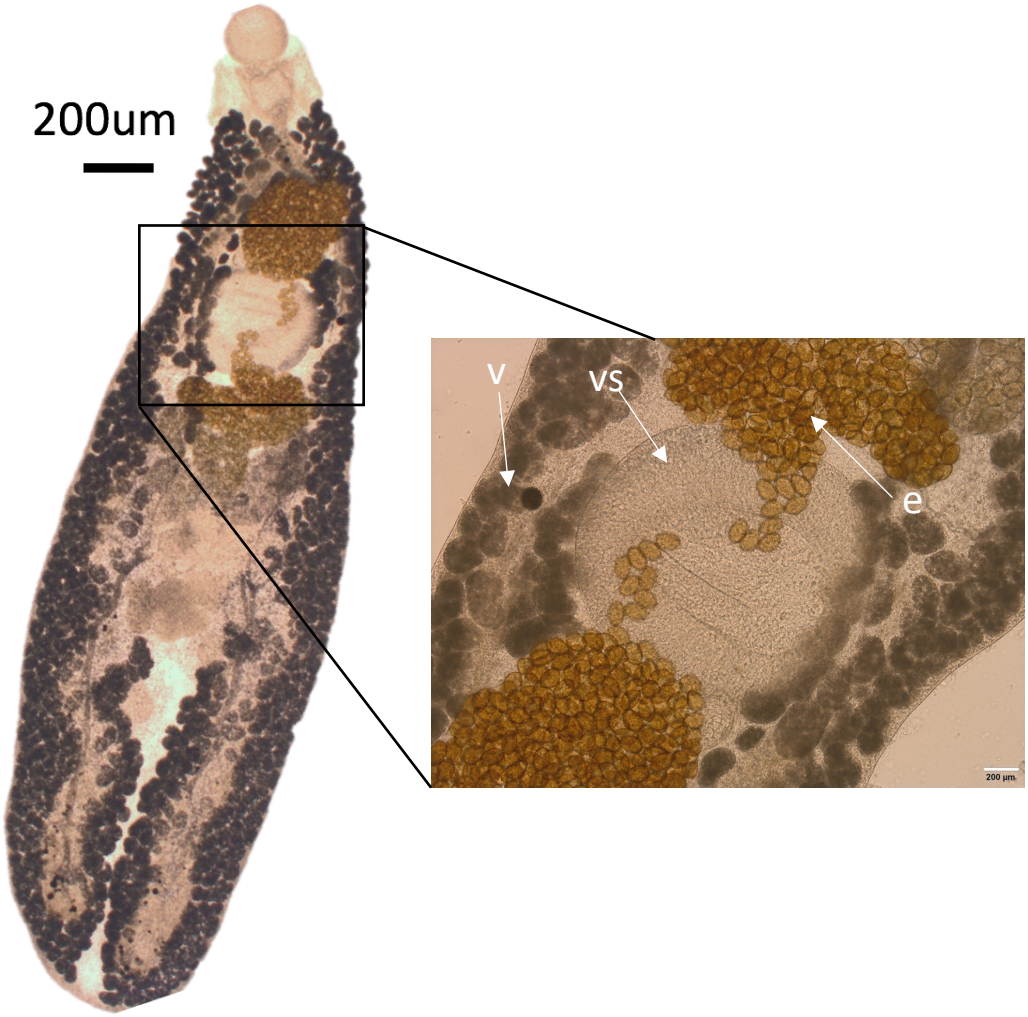

Figure B3. Digenetic trematode found in the renal sac of *Octopus huttoni*. The zoomed in frame displays the ventral sucker (vs), eggs (e), and vitellaria (v).

Figure B4. Photo of the location where most trematodes (indicated by white circle) were found in *Octopus huttoni*.

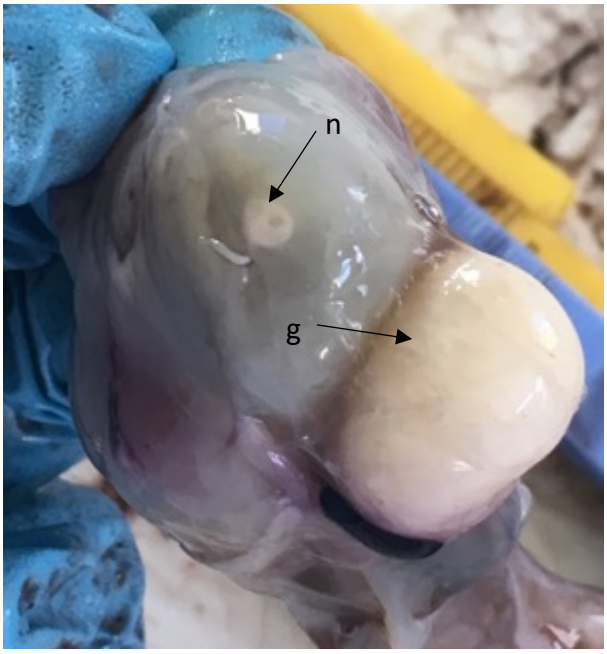

Figure B5. Nematode curled intramuscularly under the mantle near gonad in a male *Octopus huttoni* from the Otago Harbour.

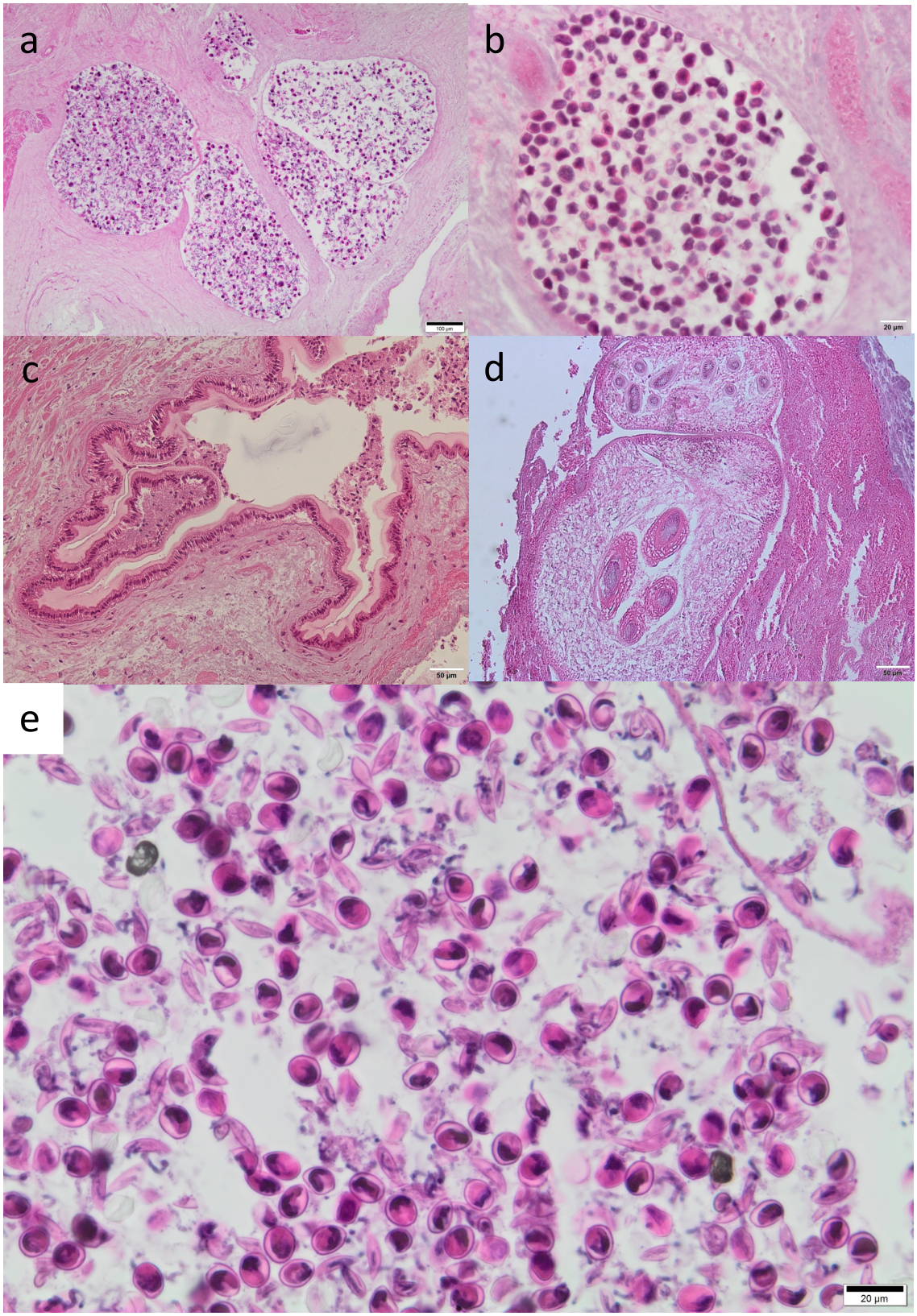

Figure B6. Micrographs of histological sections (5µm) of coccidian parasites found in connective tissue and digestive system of *Octopus huttoni* stained with hematoxylin and eosin. (a)oocysts in skin tissue (scale bar = 100 µm), (b) sporocysts in oocyst (scale bar = 20 µm), (c) sporont showing folding (scale bar = 50 µm), (d) macromeronts (scale bar = 50 µm), (e) sporocysts and sporozoites within an oocyst (scale bar = 20 µm).

Discussion

Although the coccidian parasite could not be identified because the number of sporozites per sporocyst could not be verified, it most closely resembles the apicomplexan *Aggregata sp*. which is common in other species of octopus. *Aggregata* uses crustaceans as an intermediate host in which merogony occurs and cephalopods as final hosts where gamogony and sporogony occurs (Castellanos-Martínez & Gestal, 2013; Gestal *et al.*, 1999, 2002b; Mayo-Hernández *et al.*, 2013; Mladineo & Bočina, 2007). Because many of the infected individuals were highly infested (oocysts found outside of digestive system), many of the histological photos appear to display sporogony. Highly infected senescent octopods were predominantly infected with sporogonial and a few merogonial stages that were spread throughout the tissues (Castellanos-Martínez & Gestal, 2013).

All other parasites have not been reported to have a negative impact on the host whereas *Aggregata* is known to cause malabsorption syndrome (Gestal & Castellanos-Martínez, 2015; Castellanos-Martínez & Gestal, 2013; Gestal *et al.*, 2007; Pascual *et al.*, 2007; Gestal *et al.*, 2002b, 2002a). The parasite damages the lumen in the digestive tract causing a change in pH which in turn causes the digestive enzymes to malfunction (Gestal & Castellanos-Martínez, 2015; Castellanos-Martínez & Gestal, 2013; Gestal *et al.*, 2007; Pascual *et al.*, 2007). *Aggregata* also causes necrosis, or cell death (Castillo *et al.*, 2015; Pascual *et al.*, 2010; Mladineo & Bočina, 2007; Gestal *et al.*, 2002a), which was also observed in this study.

Castillo *et al.* (2015) attributes exposure to bacterial toxins to the cause of cell death. So while the coccidian itself does not cause death, it may weaken the immune system, allowing deadly bacteria and viruses to infect the host (Castellanos-Martínez & Gestal, 2013; Pascual *et al.*, 2007; Gestal *et al.*, 2002b, 2010; Storero & Narvarte, 2013; Poynton *et al.*, 1992). If an infected host is consumed, it could also have significant health implications on the human consumer such as coccidiosis and toxoplasmosis (Suong *et al.*, 2017). These are serious infectious disease that cause malabsorption syndrome (Gestal & Castellanos-Martínez, 2015).

Although *Aggregata sp*. has not been reported in any New Zealand cephalopods, an apicomplexan has been found in the oyster *Ostrea chilensis* in Foveaux Strait, Bluff (Hine, 2002). This parasite is termed apicomplexan parasite 'x' (APX) because it does not morphologically resemble any other apicomplexan found in molluscs (Suong *et al.*, 2017). Because many of the infected individuals from this study were sourced from the Foveaux Strait, there is a possibility that the apicomplexans found in both *O. huttoni* and *O. chilensis* are the same. To further investigate this, studies on the prey preference of *O. huttoni* in the Foveaux Strait must be done to see if they prey on this species of oyster and if these apicomplexans are the same.

The trematode was identified to be *Plagioporus maorum* using Allison (1966), which is found in the Māori octopus, *Macroctopus maorum*, and the midget octopus, *O. huttoni* (Previously known as *Robsonella australis*) (Allison, 1966; Short & Powell, 1968; Kinne, 1990) . *P. maorum* is typically found in the renal sac or just adjacent such as those found in the present study (Allison, 1966; Short & Powell, 1968; Kinne, 1990). It is common in *M. maorum (*specifically individuals around Kaikoura) where more than 50 trematodes can be found at a time. *O. huttoni* is only occasionally infected (specifically individuals near Portobello) and only one worm is found at a time (Allison, 1966; Short & Powell, 1968; Kinne, 1990). 40% or more of octopods can be infected with this trematode and the presence of sexually mature adults indicates that octopods are most likely final hosts for this species (Kinne, 1990). Although occurrences of different trematodes have been recorded in a range of octopus species, there is no data on whether these worms are harming the octopus host and causing death.

The larval cestodes could be identified to the order Trypanorhyncha because of the presences of four hooked tentacles. They are considered larval because they are found in teardrop shaped cysts. Smith *et al.* (1981) has reported a post-larvae tryanorhynchid cestode *Nybelinia* in New Zealand arrow squid suggesting that the cestode found in this study could be of the same genus. *Nybelinia* also has high infestation rates in arrow squid (Smith *et al.*, 1981) indicating that it utilizes *O. hutttoni* just as much as other cephalopod species.

The nematode could not be extracted from the mantle skin or identified, but it may be an anisakid nematode, which is known to infect the mantle musculature of cephalopods (Kinne, 1990). If infected cephalopods are eaten raw, the parasite can be transmitted to consumer such as humans, which can cause ulcers or lesions (Kinne, 1990). Larvae of the anisakid nematode *Anisakis simplex* has been found in New Zealand arrow squid (Smith *et al.*, 1981), which suggests that the same species, or at least genus, is affecting *O. huttoni*.

Except the trematode, these parasites have been reported in *O. huttoni*. Nematodes and cestodes have been reported in other New Zealand cephalopods (Smith *et al.*, 1981), but not the coccidian parasite. There is a possibility that these parasites have not been reported from *O. huttoni* because they were less prevalent. Environmental change in recent years could be facilitating rapid growth of parasites and therefore increasing infection rates. Future studies should study how they use the cephalopod host and investigate how they might infect more hosts with increased ocean warming.

This recent discovery of parasites in *O. huttoni* presents data on the kinds of parasites and their prevalence. With this information, future studies can be compared to observed how changing environmental factors might be affecting parasites in *O. huttoni*. In regard to the present study, presence of parasites might further explain why midget octopus from Bluff are smaller than those in the Otago Harbour. Infected individuals from Bluff were mostly parasitized by the coccidian and larval cestodes which are found in the digestive tract. Those infected from Otago were mostly parasitized by the trematode which are found in the renal sac or adjacent. Because parasites in the Bluff individuals were found in the digestive tract and it is known that the coccidian causes malabsorption, it can be hypothesised that these parasites are causing loss of condition. Only seven individuals from Bluff were infected with the coccidian, but there is a possibility that more were infected, but the coccidian was too small to see macroscopically.

Appendix C: Marine heat waves

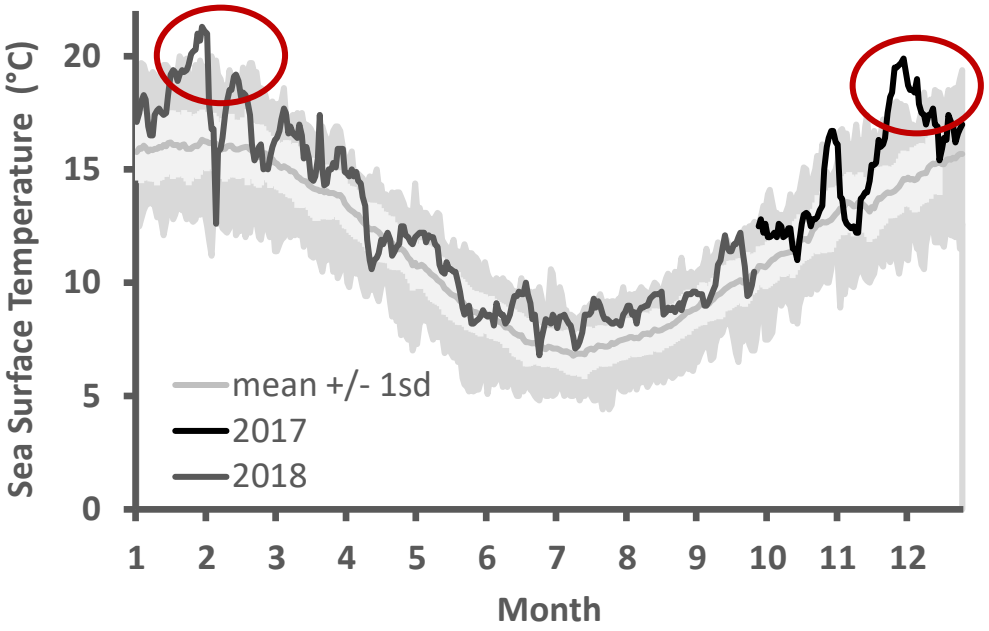

Figure C1. Sea surface temperature (°C) measured from Portobello Marine Lab Wharf every day from 1953 to 2016 where the grey line is the mean, the dark shaded areas are the range, the black line is data from 2017 the dark grey line is the data from 2018, and the red circles indicate the marine heat wave.